Vol. 6. No. 3. SEPTEMBER 1951

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# BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY

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THE BRITISH PHARMACOLOGICAL SOCIETY
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A. D. MACDONALD J. W. TREVAN E. J. WAYNE
J. H. GADDUM (Chairman) H. R. ING (Press Editor)
In co-operation with the EDITOR of the British Medical Journal

LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, W.C.1

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*Brit. J. Pharmacol.* (1951), **6**, 351.

# A PRESSOR SUBSTANCE IN URINE

BY

J. DEKANSKI

*From the Pharmacology Department, University of Edinburgh, and Research Department, Bristol Mental Hospitals*

(Received November 2, 1950)

The gonadotrophins in human urine may be concentrated by adsorption on kaolin (Scott, 1940; Dekanski, 1949, 1950). A substance resembling vasopressin is present in the supernatant fluid, after the gonadotrophins have been removed, and may be concentrated by the method developed by Grollman and Woods (1949) for the concentration of the urinary antidiuretic factor. The pressor activity of these concentrates was assayed in comparison with vasopressin on rats' blood pressure (Landgrebe, Macaulay, and Waring, 1946), the method being eventually modified in certain details.

## METHODS

*Extraction of urine.*—The total amount of urine collected during 24 hours was first treated with kaolin. The acidic supernatant fluid was partly siphoned off when the kaolin had settled down and partly poured off after the kaolin suspension had been centrifuged. This acid fluid was filtered through a Buchner funnel under reduced pressure and treated by the method used by Grollman and Woods (1949) for the concentration of the antidiuretic fraction.

*Assay methods.*—Assays were carried out for pressor, antidiuretic, and oxytocic effects.

The pressor activity of urinary concentrates was quantitatively assayed on rats' blood pressure by a method similar to that of Landgrebe, Macaulay, and Waring (1946). The best results were obtained with rats weighing between 300 and 350 g., anaesthetized with urethane, injected subcutaneously, and treated with dibenamine (Nickerson, 1949; Nickerson and Goodman, 1947). The animals were kept warm and were ready for the experiment 45–60 minutes after the injection of urethane. The trachea was cannulated in the usual fashion and one carotid artery ligated. The femoral vein was then cannulated just below the inguinal ligament, the corresponding artery separated, and the posterior division of the femoral nerve cut. A blunted needle (No. 1) was used for venous cannulation, and heparin was injected intravenously. All injections were made through a two-way stopcock fitted with a tuberculin syringe, and all solutions were washed in with 0.2 c.c. normal saline from a microburette (with attached thistle funnel) connected with a piece of rubber tube. The carotid cannula was connected with a mercury manometer by a column of normal saline. The C.N.S. was left intact, in order to avoid excessive bleeding after heparin, the vagi and associated sympathetics being cut at the end of the operation. Dibenamine was injected intravenously just before the beginning of assay.

On two occasions the antidiuretic activity of the same concentrates was assayed by measuring the average antidiuretic response in groups of rats (Burn, 1931), Pitressin

(Parke, Davis and Co.) being used as the standard preparation for both the pressor and antidiuretic assays. Oxytocic activity was assayed on the virgin guinea-pig's uterus (Dale and Laidlaw, 1912) suspended in a bath containing a salt solution with no Mg (Burn, 1937), Pitocin (Parke, Davis and Co.) being used as the standard preparation.

*Drugs used :*

*Intraperitoneally :* Allobarbitone—Dial Liquid Comp. (Ciba)—0.03 c.c./100 g. body weight (followed by urethane injections).

*Subcutaneously :* Urethane (B.D.H.)—25 g. per cent (w/v) solution in distilled water—0.7 c.c./100 g. body weight.

*Intravenously :* Heparin *B.P.* (Boots), sterile, 109.3 u./mg.—2 mg./100 g. body weight dissolved in 1–2 c.c. normal saline, *l*-adrenaline base (B.W. and Co.) and *l-nor*adrenaline bitartrate (Bayer). Doses given in mμg. of the base dissolved in normal saline containing ascorbic acid ($5 \times 10^{-3}$). Piperidine acid tartrate (B.D.H.)—100 mg. per cent (w/v) solution in normal saline (≡36.3 mg. base per 100 c.c.). Ergotoxine ethanesulphonate (B.D.H.)—25 mg. per cent (w/v) suspension in faintly alkaline saline. Piperoxane HCl (May and Baker) (piperidylmethylbenzodioxane hydrochloride, 933F) —0.2 mg./c.c. solution in normal saline. Dibenamine HCl (Smith, Kline, and French) (N : N-dibenzyl-β-chloroethylamine)—2.5 mg./c.c. solution in faintly acid saline; 12.5 mg. dibenamine HCl dissolved in 0.5 c.c. 95 per cent ethanol made acid (0.05 N) with concentrated $H_2SO_4$ and then diluted up to 5 c.c. with normal saline.

## Results

The pressor equivalents are given in Table I. When the standard preparation was injected in small doses (4–20 mu.) there was always a simple pressor effect, as seen in other anaesthetized mammals, but with very high doses a diphasic response may occur when coronary vasoconstriction is severe enough to affect the heart. There was never enough of the pressor factor in a 24-hour specimen to cause such a diphasic response. The intravenous injections of the concentrates were interposed between the injections of the standard, doses producing effects

TABLE I

Individual results. Pressor equivalents (mu.) per 24-hour urinary specimens. Concentrates diluted with normal saline.

| Subject— Age, Sex (M) = male (F) = female | 24-hour urinary output (c.c.) | Final volume of concentrate (c.c.) | Volume (c.c.) of concentrate equivalent to 0.004 i.u. pitressin | Total pitressin equivalent (mu.) |
|---|---|---|---|---|
| P. 67 (F) .. .. | 1,640[1] | 10 | 0.35 | 114 |
| F. 26 (F) .. .. | 1,800[1] | 10 | 0.4 | 100 |
| D. 52 (M) .. .. | 1,550 | 5 | 0.2 | 100 |
| D. 52 (M) .. .. | 1,200 | 5 | 0.25 | 80 |
| B. 30 (M) .. .. | 1,560 | 5 | 0.25 | 80 |
| B. 23 (F) .. .. | 1,400 | 5 | 0.25 | 80 |
| D. 52 (M) .. .. | 1,100 | 5 | 0.25 | 80 |
| D. 52 (M) .. .. | 580[2] | 5 | 0.15 | 133 |
| S. 22 (F) .. .. | 1,120[3] | 20 | 0.25 | 320 |
| S. 22 (F) .. .. | 1,200 { 600 / 600[4] | 2.5 / 50 | 0.25 / 0.25 | 40 / 800 |

[1] Concentrate tested also for antidiuretic effect. [2] Urine collected during 36 hours' withdrawal of food and water. [3] 0.5 i.u. pitressin added to supernatant urine (*p*H 4.0). [4] 1 i.u. pitressin added to 600 c.c. of supernatant urine (*p*H 4.7), the other 600 c.c. being used as control.

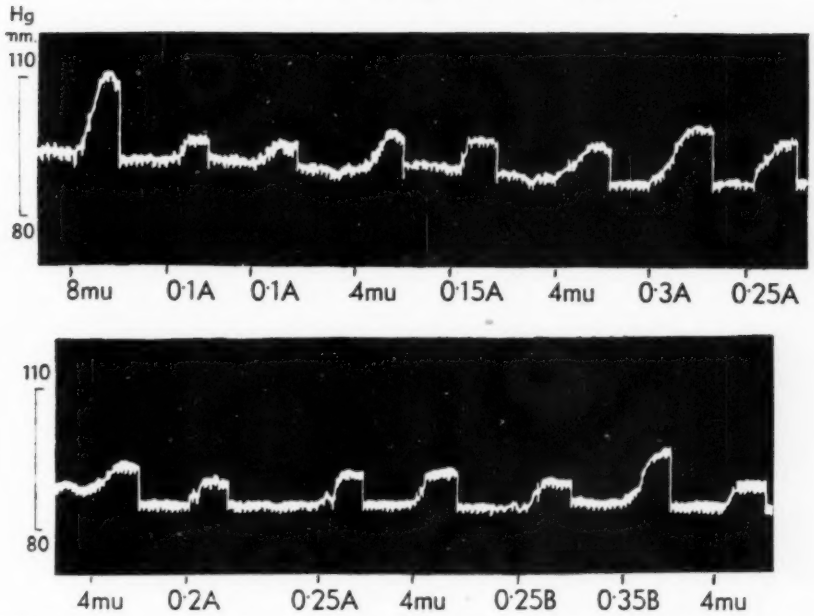


FIG. 1.—Effect of urinary pressor fractions A and B compared with that of pitressin on rat's carotid blood pressure. 10-minute intervals. Doses in mu. (4 mu. in 0.1 c.c.) and c.c. A = a concentrate (× 12) of urine with added pitressin (1,000 mu. per 600 c.c.). B = a concentrate (× 240) of the same urine without pitressin.

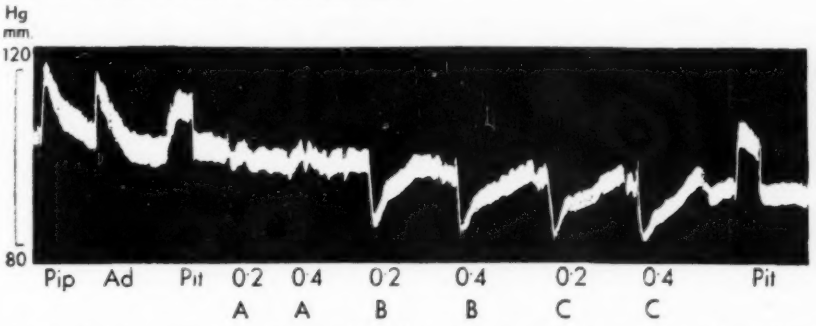


FIG. 2.—Rat's blood pressure. Pip = piperidine (110 μg. base). Ad = adrenaline (0.6 μg.). Pit = pitressin (10 mu.). A, B, and C—concentrates (× 200) made by method of Grollman and Woods. Doses in c.c. A = piperidine (90 mg.) in 1 l. water. B = piperidine (90 mg.) in 1 l. decomposed urine. C = same urine alone. If piperidine had survived the concentration A and B would have contained 18 mg. per c.c.

above and below that of the standard being determined (see Fig. 1). The concentrates of unpreserved urine which had been left for a few days showed a definite depressor effect (see Fig. 2), and it was found necessary to extract fresh specimens only.

When one unit of pitressin was added to 600 c.c. of the supernatant fluid after removal of the kaolin, 76 per cent of it was recovered by the methods used (Table I, Fig. 1). In another experiment the recovery was about 50 per cent. When piperidine was similarly added to water or the supernatant fluid, none of it was recovered (Fig. 2).

The pressor effect of urinary concentrates may be due to the presence of nicotine, piperidine, and other pressor bases. Therefore, the identification of the urinary pressor factor was carried out by testing it on rats treated with ergotoxine (30–40 μg./100 g. body weight), piperoxane (5–10 μg./100 g. body weight), or dibenamine (1–2 mg./100 g. body weight). Both ergotoxine and piperoxane reverse the pressor action of adrenaline in the rat, as they do in the cat, but the urinary pressor fraction, like pitressin, still caused a rise of blood pressure after these drugs had been given. However, these results were complicated by the toxic effects of ergotoxine and by the short duration of the action of piperoxane, and often by the incompleteness of the blocking action of those compounds. Dibenamine hydrochloride, on the other hand, was found to have definite advantages of specificity, potency, and prolonged action.

The total dose of 1–2 mg. dibenamine hydrochloride per 100 g. body weight was injected intravenously into the rat in five equal portions, spread over 25–30 minutes. Rapid injection caused a marked fall in arterial pressure; even a slow rate of injection usually produced a progressive fall in blood pressure, but this was not fatal. If necessary, artificial respiration was used. The blood pressure soon established itself on a new basal level and remained constant for seven to nine hours.

After the administration of dibenamine, the usual pressor effects of small doses of adrenaline, *nor*adrenaline, and piperidine were always abolished, but never changed to a pure depressor response even when the total dose of injected dibenamine was as much as 4 mg./100 g. body weight. The pressor response to pitressin and the urinary pressor fraction, on the other hand, still occurred after

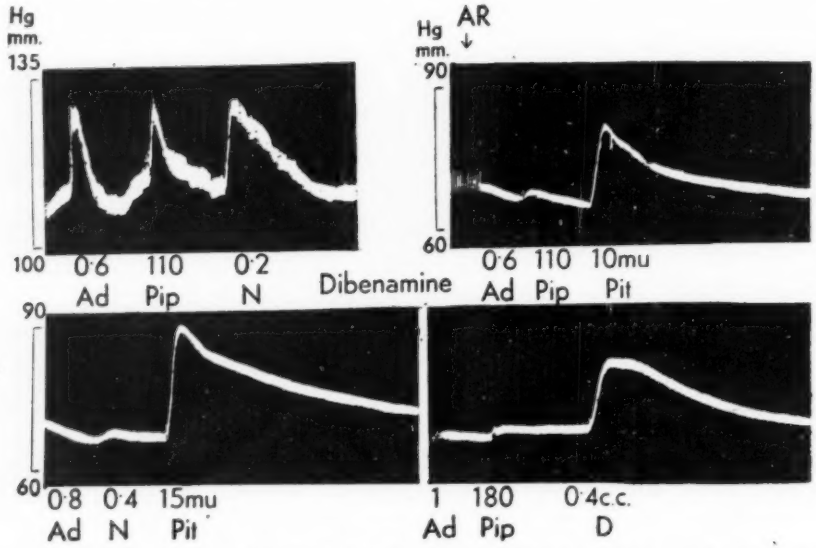


FIG. 3.—Rat's blood pressure. Dibenamine (1 mg./100 g. body weight) injected after the first three records had been taken caused a fall of B.P.; artificial respiration was started at this time. The effects of adrenaline (Ad) *nor*adrenaline (N), and piperidine (Pip) were abolished by dibenamine (doses in μg.), but the effects of pitressin (Pit) or of a concentrate (× 240) of urine (D) were not.

dibenamine (Fig. 3), and in good preparations 40 or more satisfactory responses were obtained. It was found that useful discrimination for the assay of pituitary pressor hormone was obtained with doses of about 4 to 20 mu. With doses of 60 mu. and over the responses were less regular, even with intervals of 15 minutes between injections. The maximum simple pressor response occurred with a dose of 200 mu. of pitressin.

The antidiuretic effect of urinary concentrates was quantitatively assayed on two occasions in comparison with the pressor effect. When injected subcutaneously into the rats, two fractions produced antidiuretic responses equivalent to about 50 mu. and 80 mu. of pitressin per 24-hour specimen. These figures were 50 and 70 per cent of the estimates of the pressor activity in the same solutions.

The average amount of pressor activity recovered from the urine excreted in 24 hours was equivalent to about 90 mu. of pitressin (Table I). The withdrawal of water appeared to be associated, in one experiment, with a small increase in the amount of pressor activity found in urine.

No significant oxytocic activity was detected in the concentrates examined. In one experiment the concentrate from 240 c.c. urine contained the equivalent of less than 3 mu. pitocin. The pressor activity of this fraction was equivalent to 80 mu. pitressin.

## Discussion

Previous work has shown that several pressor substances may be present in extracts of urine. Fresh urine has been shown to contain adrenaline, *nor*adrenaline, and hydroxytyramine (Holtz, Credner, and Kroneberg, 1947 ; Holtz, 1950 ; Euler, 1950), nicotine (Dingemanse and Freud, 1933 ; Helmer, Kohlstaedt, and Page, 1939 ; Lockett, 1944a, 1944b), and piperidine (Euler and Sjöstrand, 1943 ; Euler, 1944a, 1944b, 1945). Bacterial action may cause the formation of *iso*amylamine (Bain, 1915 ; Bohn and Hahn, 1933 ; Bohn and Coester, 1934 ; Lockett, 1944a) and tyramine (Enger and Arnold, 1937). All these bases have pressor actions, but the experiments with ergotoxine, piperoxane, and dibenamine have shown that the pressor substance described here is not identical with any of them. Direct experiment has shown that any piperidine present originally would not be present in the final concentrates. The substance studied in these experiments is probably the pressor principle from the posterior pituitary, vasopressin, which has indeed been previously found in urine. For example, Dale (1909) showed that when posterior pituitary extracts were injected intravenously in cats the pressor principle could be detected in the urine by its effect on the cat's blood pressure. There has also been much recent work on the excretion of the antidiuretic principle in the urine. This substance is thought (Waring and Landgrebe, 1950) to be identical with the pressor principle, and assays based on the antidiuretic effect should therefore give the same result as assays based on the pressor effect. The results of preliminary experiments on these lines, with the urinary concentrates obtained by the method described, are suggestive but by no means conclusive. The test for oxytocic activity, on the other hand, gave negative results and showed that oxytocin was not present in comparable amounts.

SUMMARY

1. Human urine, from which gonadotrophins had been removed with kaolin, was treated by the method of Grollman and Woods for the preparation of antidiuretic concentrates. These concentrates were then tested for other forms of activity.

2. They had a pressor action in rats which was not due to adrenaline, *nor*adrenaline, *iso*amylamine, tyramine, piperidine, or nicotine, since it survived the injection of dibenamine, ergotoxine, or piperoxane (933F). It is thought that the effect was due to vasopressin.

3. The urine concentrates appeared to have no action on the guinea-pig's uterus. Their oxytocic activity in terms of posterior pituitary extracts was certainly less than 1/20th of their pressor activity.

I wish to thank Professor J. H. Gaddum and Dr. Max Reiss for their valuable help and advice, and also Organon Laboratories, Ltd., for providing facilities for this work.

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*Brit. J. Pharmacol.* (1951), **6**, 357.

# CYTOTOXIC AGENTS: III, DERIVATIVES OF ETHYLENEIMINE

BY

J. A. HENDRY, R. F. HOMER, AND F. L. ROSE

*From Imperial Chemical Industries, Ltd., Research Laboratories, Hexagon House, Manchester, 9*

AND

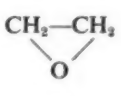
A. L. WALPOLE

*From Imperial Chemical Industries, Ltd., Biological Laboratories, Wilmslow, Cheshire*

(Received March 30, 1951)

In Parts I and II of this series (Hendry, Rose, and Walpole, 1951 ; Hendry, Homer, Rose, and Walpole, 1951) we described the cytotoxic and tumour-inhibitory properties exhibited by certain polymethylolamide and bis-epoxide derivatives. Substances of these types were selected because of their use in textile technology in modifying the properties of fabrics. They are believed to bring about their peculiar effects by the formation, by virtue of their polyfunctional reactivity, of covalent linkages across the cellobiose or peptide chains of the textile fibres. It seemed not unlikely that the specific biological effects of these compounds were manifestations of a similar behaviour within the cell, and it was suggested that here the affected system was either protein or nucleoprotein, possibly of nuclear origin, since chromosome damage in dividing cells seemed to underlie the more significant features of their action.

Compounds of a third chemical type were known to us to have applications similar to those of the methylolamides and epoxides in the textile field, and indeed were already under investigation in that connexion by our colleagues in these laboratories. These compounds were based on ethyleneimine (II), which in its chemical reactivities closely resembles the analogous ethylene oxide (I) molecule. The simple parent substance, like ethylene oxide, itself produces modifications in the physical properties of fibres, but the most effective agents in this series contain two or more imine residues, just as the most useful epoxides contain two epoxide groups. It is perhaps worthy of note at this point that the most convenient mode of combination of the imine residues in the molecule is usually through a linkage at the nitrogen atom (III), whereas the epoxide groups must necessarily be combined through a group attached to one of the carbon atoms (IV).



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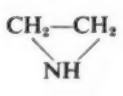

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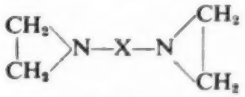

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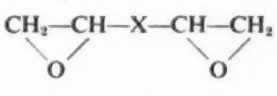
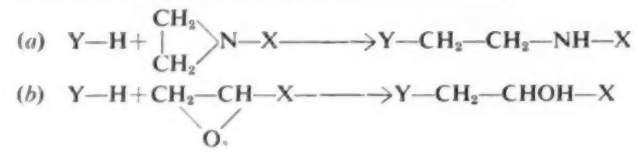


(IV)

Nevertheless it is found that N-substituted ethyleneimines and C-substituted ethylene oxides show a like reactivity towards nucleophilic reagents, leading to chemical union with the second reactant according to the schemes:



The methylolamides described in Part I behave similarly according to the scheme:

Well over a hundred derivatives of ethyleneimine have been prepared and subjected to a variety of biological tests during the past two years, so that it has become possible to relate structure with effect fairly closely. As already indicated, some of the compounds were derivatives that had been initially conceived as textile auxiliary agents. These included the early 1 : 3 : 5-triazine derivative, 2 : 4 : 6-tris-ethyleneiminotriazine (Serial No. 322), certain bis-ureas (276, *et seq.*), and bis-sulphonamides (287–294).

At an early stage it became apparent that, with a few notable exceptions, marked cytotoxic and tumour-inhibitory action in the animal was dependent upon the presence within the drug molecule of at least two ethyleneimine residues, and the chemical aspects of the research (to be recorded elsewhere) became essentially an investigation of the many possible ways in which this could be brought about. Since ethyleneimine is a dialkylamine, the preparation of amide structures was an important theme, not least because the necessary products could be obtained at temperatures below those at which secondary reactions intervened. The facility with which condensation occurs between the amine and chloroheterocyclic structures was also exploited. Thus, with cyanuric chloride as starting material, the three chlorine atoms could be replaced stepwise more or less at will by one or two ethyleneimine residues and other amine or alkoxy groupings* (compare 272, 314, 320, and 321). Similarly, in the guanamine series,* it was possible to prepare compounds such as 324 and 325. Di- and trichloropyrimidines were utilized in the same way,* although with these the necessary reactivity towards ethyleneimine could be obtained only by having present certain other substituents in the pyrimidine nucleus (compare 261, 269, 299, 304, etc.).

As the investigation developed, isolated instances were found of monoethyleneimine derivatives having tumour-inhibitory activity, for example, 2 : 4-dinitrophenylethyleneimine (257), and attempts to elucidate what seemed to be the peculiar mode of action of this compound led to the preparation of related substances such as 259, 335, 337, and 342.

During the course of this research it was learned that workers at the Sloan-Kettering Institute, New York, had independently come to examine substances based on ethyleneimine, including a number formulated in the Tables below. It is a pleasure to record here the valuable exchange of notes and discussion that has followed this coincidental discovery.

* Patent protection pending.

## Experimental

*Toxicity.*—Toxicity tests in mice and rats were carried out on all compounds, given intraperitoneally, as described in an earlier paper (Hendry, Rose, and Walpole, 1951). The majority were either dissolved in water or, if insoluble, suspended by milling in an aqueous solution of Dispersol OG, 1.5 per cent, and Dispersol LN, 0.05 per cent. The remainder were dissolved or suspended in arachis oil. Solutions and suspensions were kept in the refrigerator; where marked instability was known or suspected they were made up freshly before injection. Ethyleneimine was kept over caustic pellets and dissolved in $CO_2$-free water immediately before use. The results of these tests are not recorded here, but from them an estimate was made of the highest doses that could be given to rats daily, Sundays excepted, for the first 10 to 12 days after implantation of the Walker tumour, without any of the animals dying by the fourteenth. This schedule of dosage was used in initial tests for tumour-inhibitory activity.

*Tumour inhibition.*—Our standard method of test for inhibitory activity, using the Walker tumour, has also been described (Walpole, 1951) and was briefly as follows.

Single subcutaneous implants were made in stock rats of about 100 g. in weight, and the rats were then separated into groups of from 10 to 15. One group was kept untreated as control and the others dosed as above with the compounds under test. On the fourteenth or, occasionally, the fifteenth day—the day of implantation being taken as day 0—all the animals surviving in the experiment were weighed and killed and the tumours dissected out and weighed. The percentage increase in gross weight ($\triangle W$) of the survivors in each group and the percentage inhibition of tumour growth ($I$) in each of the treated groups was then calculated, the latter according to the formula.

$$I = \frac{(M_{50}\ \text{controls} - M_{50}\ \text{treated})}{M_{50}\ \text{controls}} \times 100$$

where $M_{50}$ was the mean weight of the $n$ largest tumours in any group of $2n$.

## Results

In Tables I to III are listed all the compounds examined. The serial and code numbers, and the name and formula of each, are shown together with the total (intraperitoneal) dose in mg. per 100 g. rat given over the first 10 to 12 days after implantation of the tumour. Compounds will be referred to in the sequel either by name or by serial number or both.

Table I includes ethyleneimine itself (Serial No. 246), polyethyleneimine (246a), and a number of monoethyleneimine derivatives (247–274). Many of these are inactive or inhibit tumour growth no more than might be expected from their general "toxic" action. A few, however, are active in the sense that with them the inhibition produced is greater than can be accounted for on that basis. Of these the dimethoxytriazine 272 has already been reported by workers at the Sloan-Kettering Institute [see Note in review by Philips (1950)] as showing appreciable tumour-inhibitory and cytotoxic activity. To this must now be added other heterocyclic monoethyleneimines, notably the corresponding diaminotriazine 271, the dichloropyrimidine 262, and the phenyldichloropyrimidine 263. With all these activity is comparatively low, being no more than one-tenth to one-hundredth that of the nearest related bis-ethyleneimine derivatives, and the methods of preparation were such that the possibility cannot be excluded that the latter were present as contaminants in amount sufficient to produce the observed biological effects. Great

TABLE I

ACTION OF SOME MONO-FUNCTIONAL ETHYLENEIMINE DERIVATIVES UPON THE WALKER TUMOUR IN RATS

a.s., aqueous solution; o.s., oily solution; a.d., aqueous dispersion; o.d., oily dispersion; $\triangle W$, mean percentage increase in gross weight of tumour-bearing rats; $M_{50}$, mean weight of $n$ heaviest tumours in groups of $2n$; $I$, percentage inhibition of tumour growth; L, sample

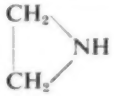
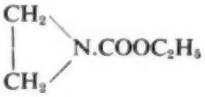
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| 246 | 10355 | Ethyleneimine |  | a.s. ($CO_2$-free water) | 0.5 |
| 246a | 9162 | Polyethyle n | $[-CH_2CH_2NH-]_n$ | a.s. | 8.5 |
| | | AMIDES | | | |
| 247 | 9209 | Acetylethyleneimine | | ,, | 13 |
| 248 | 9952 | Stearoylethyleneimine | | | Untested |
| 249 | 9210 | Benzoylethyleneimine | | o.s. | 10 |
| 250 | 10577 | *p*-Nitrobenzoyl-ethyleneimine | | a.d. | 15 |
| 251 | 9211 | *p*-Toluene sulphonyl-ethyleneimine | | ,, | 52.5 |
| 252 | 10755 | *p*-Nitrobenzene sulphonylethylene-imine | | ,, | 47.5 |
| | | URETHANES AND UREAS | | | |
| 253 | 10356 | Carbethoxyethylene-imine |  | o.s. | 4 |
| 254 | 9212 | Phenylcarbamyl-ethyleneimine | | a.d. | 20 |

TABLE I (*continued*)

limited in amount, maximal dose not given; *A*, percentage anaphases in excess of controls showing specific chromosome effects in tumour tissue from rats bearing the Walker tumour, 24 hours after the doses shown; *B*, true chromosome bridges; *D*, degenerating cells; *E*, "exploded" nuclei; *F*, chromosome fragments; *M*, accessory micronuclei; *P*, pyknotic nuclei; *S*, "sticky" chromosome bridges

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | *A* | Remarks | |
| 15.0 | −3.2 | 29.1 | 21.8 | 25 | 0.5 | 4 | | A few *S* |
| 27.7 | 31.0 | 31.6 | 26.7 | 16 | — | — | | — |
| 35.1 | 13.4 | 36.1 | 27.5 | 24 | — | — | | — |
| | | | | | 20 | 9 | | Normal |
| 35.4 | 13.2 | 37.9 | 34.4 | 9 | — | — | — | — |
| 45.7 | 28.4 | 42.5 | 36.7 | 14 | 5.0 | 3 | | A few *S* |
| 35.4 | 38.7 | 37.9 | 43.5 | 0 | — | — | — | — |
| 34.6 | 24.6 | 46.1 | 35.3 | 23 | 8.0 | 4 | — | Normal |
| 39.7 | 18.1 | 49.4 | 35.5 | 28 | 4 | 3 | — | A few *S* and *P* |
| 35.4 | 18.8 | 37.9 | 41.6 | 0 | — | — | — | — |

TABLE I (*continued*)

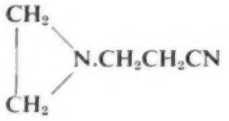
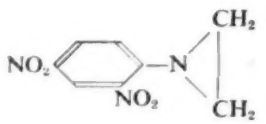
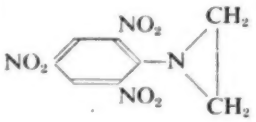
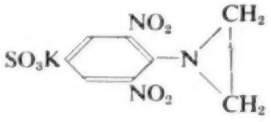
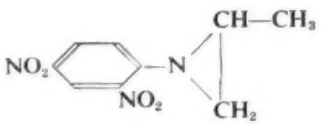
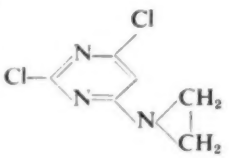
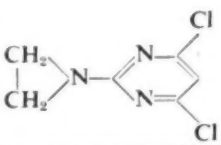
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| | | NITRILES AND ESTERS | | | |
| 255 | 10574 | β-Ethyleneimino-propionitrile |  | a.s. | 7.2 |
| 256 | 9213 | γ-Ethyleneimino-propyl methacrylate | | o.s. | 29 |
| | | NITROPHENYL DERIVATIVES | | | |
| 257 | 10200 | 2 : 4-dinitrophenyl-ethyleneimine |  | a.d. | 25 |
| 258 | 10357 | 2 : 4 : 6-trinitrophenyl-ethyleneimine |  | ,, | 40 |
| 259 | 10425 | 2 : 6-dinitrophenyl-ethyleneimine-4-sulphonic acid (K salt) |  | ,, | 375 |
| 260 | 10800 | N-2 : 4-dinitrophenyl-C-methyl-ethyleneimine |  | ,, | 130 |
| | | PYRIMIDINES | | | |
| 261 | 9605 | 2 : 4-dichloro-6-ethyleneimino-pyrimidine |  | o.d. | 9.5 |
| 262 | 11154 | 4 : 6-dichloro-2-ethyleneimino-pyrimidine |  | a.d. | 100 |

TABLE I (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 52.4 | 16.3 | 32.3 | 17.8 | 45 | 2 | 4 | (Mean of 2 results) | A few *S* |
| 35.1 | 29.9 | 36.1 | 35.0 | 3 | — | — | — | — |
| 18.8 | −10 | 35.7 | 0.8 | 98 | 10 | 60–64 | Some inhibition of mitosis. Many *D* and *E* | *B* and *F* and a few *D* and *P* |
| | | | | | 5 | | Mitosis inhibited. *D*, *E*, and *M* | A few *B* and *F* |
| | | | | | 2.5 | 52 | | A few *B*, *F*, and *S*; some *D* and *P* |
| 16.6 | 13.0 | 30.5 | 30.4 | 0 | 20 | 35 | | A few *B*, *F*, and *S* |
| | | | | | 5 | 6 | | Normal |
| 24.6 | 19.1 | 23.0 | 9.4 | 59 | 20 | 53 | | A few *S* |
| 43.6 | 12.0 | 46.0 | 24.0 | 48 | 30 | 3 | | Normal |
| 42.6 | 27.1 | 42.3 | 30.7 | 27 | 2 | 23 | | ,, |
| | | | | | 1 | 12 | | ,, |
| 37.0 | 23.0 | 37.0 | 9.8 | 74 | 15 | 53 | | A few *B* and *F* |

TABLE I (*continued*)

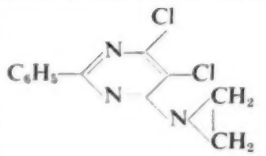
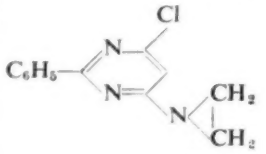
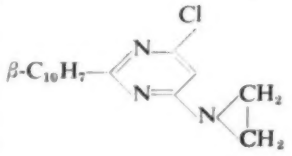
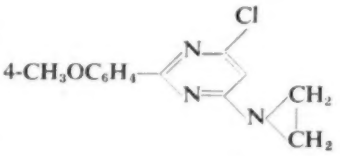
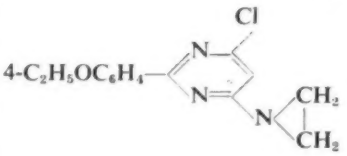
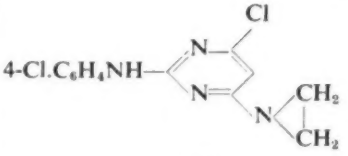
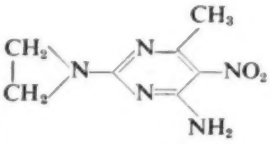
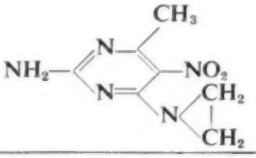
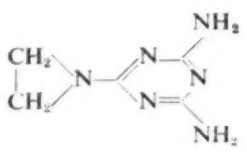
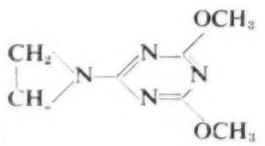
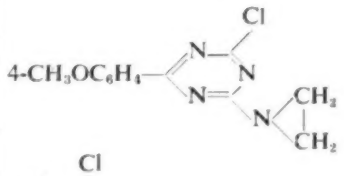
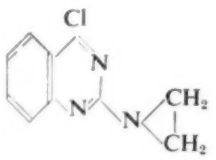
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| 263 | 11126 | 4: 5-dichloro-6-ethyleneimino-2-phenylpyrimidine |  | a.d. | 100 |
| 264 | 10038 | 4-chloro-6-ethyleneimino-2-phenylpyrimidine |  | ,, | 26 |
| 265 | 10165 | 4-chloro-6-ethyleneimino-2-β-naphthylpyrimidine |  | ,, | 15 |
| 266 | 10407 | 4-chloro-6-ethyleneimino-2-*p*-anisylpyrimidine |  | ,, | 26 |
| 267 | 10166 | 4-chloro-6-ethyleneimino-2-*p*-phenetylpyrimidine |  | ,, | 25 |
| 268 | 10036 | 4-chloro-6-ethyleneimino-2-*p*-chloranilinopyrimidine |  | ,, | 25 |
| 269 | 9951 | 5-nitro-4-amino-2-ethyleneimino-6-methylpyrimidine |  | ,, | 10 |
| 270 | 10427 | 5-nitro-2-amino-4-ethyleneimino-6-methylpyrimidine |  | ,, | 13.5 |

TABLE I (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 37.0 | 3.6 | 37.0 | 2.0 | 95 | 10 | 39 | | A few *B* and *F* |
| 37.1 | 26.7 | 40.8 | 36.1 | 12 | 8 | 6 | A few *D* | Normal |
| 31.8 | 11.5 | 46.7 | 25.3 | 46 | 2 | 0 | | A few *S* |
| 45.6 | 37.7 | 42.9 | 44.0 | 0 | 5 | 2 | | Normal |
| 18.7 | 8.7 | 35.7 | 27.8 | 23 | 10 | 0 | | A few *S* |
| 18.7 | −4.8 | 35.7 | 12.1 | 66 | 20 | 0 | | ,, |
| 45.7 | 22.8 | 42.3 | 32.1 | 25 | 0.5 | 5 | | No specimen |
| 23.2 | 8.2 | 34.3 | 17.0 | 50 | 5 | 2–4 | | A few *S* |

TABLE I (*continued*)

| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| | | 1 : 3 : 5-TRIAZINES | | | |
| 271 | 9609 | 2 : 4-diamino-6-ethyleneimino-1 : 3 : 5-triazine |  | a.d. | 9.0 |
| 272 | 10876 | 2-ethyleneimino-4 : 6-dimethoxy-1 : 3 : 5-triazine |  | ,, | 35 |
| 273 | 10304 | 2-chloro-4-ethyleneimino-6-*p*-anisyl-1 : 3 : 5-triazine |  | ,, | 6 |
| | | QUINAZOLINES | | | |
| 274 | 10307 | 4-chloro-2-ethyleneiminoquinazoline |  | ,, | 40 |

care was exercised in the isolation and purification of these monofunctional compounds, but their instability and similarity in general physical and chemical properties to related polyfunctional derivatives raised serious difficulties, while the detection of likely impurities by elementary chemical analysis was beyond the limits of error of the methods routinely employed. Set against the likelihood of contamination is the constancy of the effects produced by successive preparations of the same substance—an observation inconsistent with the expected variability in composition. An additional possibility is that, in solution, some disproportionation of the mono-compounds to bis-ethyleneimine derivatives occurs. Chemical evidence on this point has not been sought.

These arguments apart, tumour-inhibitory activity has now been found in 2 : 4-dinitrophenyl-ethyleneimine, 257, which on account of its mode of preparation and ease of crystallization must be regarded as essentially pure. This result in particular, together with certain observations upon carcinogenic activity described below, compels acceptance of the occurrence of activity in monofunctional derivatives. Further consideration of this fact is deferred to a later section.

TABLE I (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 44.1 | −5.7 | 44.1 | 6.6 | 85 | 5<br>2.5 | | Mitosis completely inhibited | Severe damage, many *D* and *P*. Moderate damage, some *D* and *P* |
| | | | | | 1.25 | 52 | Mitosis slightly inhibited | A few *B* and *F* |
| 33.0 | 4.7 | 30.5 | 0.96 | 97 | 15<br>7.5 | | Mitosis almost completely inhibited. Many *D* and a few *F* | Some *F* and *D* |
| 32.7 | 0.3 | 37.8 | 14.2 | 62 | 1.0 | 63 | Some inhibition of mitosis and some *D* and *E* | A few *B* and *S* and some *F* |
| 20.8 | 22.8 | 34.6 | 29.3 | 15<br>L | — | — | — | — |

The preparation of the dinitro-sulphonic acid derivative 259 was called for in view of a possible alternative explanation of the activity of 2 : 4-dinitro-phenyl-ethyleneimine in which the *in vivo* reduction of the *p*-nitro group to azoxy or azo with the consequent formation of a bis-ethyleneimino-azoxy- or azo-benzene was visualized. Since the *p*-sulphonic acid is active this possibility is largely ruled out.

Table II comprises compounds containing two or more ethyleneimine residues, and it will be seen that, with some few exceptions, tumour-inhibitory activity of the more specific type is consistently present throughout. Among the most effective substances are the bis- and tris-ethyleneimino derivatives from pyrimidine and from 1 : 3 : 5-triazine. Because of preparative difficulties comparison between precisely analogous members of these two series was possible in only a few instances. The most active substance of all is tris-ethyleneimino-*s*-triazine, 322, while replacement of one ethyleneimine group in this by dimethylamino gives a compound, 321, of comparable activity, suggesting that the third imine residue is superfluous. The pyrimidines analogous to these triazine compounds could not

TABLE II

ACTION ON THE WALKER TUMOUR OF SOME DERIVATIVES CONTAINING TWO OR MORE ETHYLENEIMINE GROUPS

Conventions as in Table I

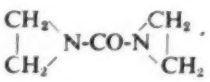
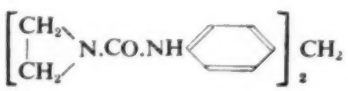
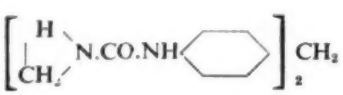
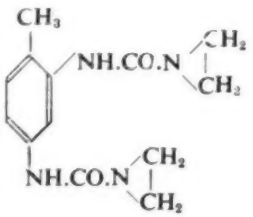
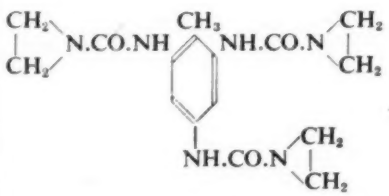
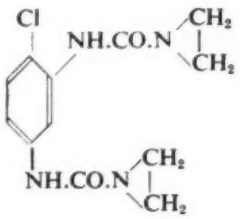
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| | | UREAS | | | |
| 275 | 10358 | N: N′-bis-*cyclo*ethylene urea |  | a.s. | 2.4 |
| 276 | 9214 | N: N′-bis-*cyclo*ethylenecarbamylhexamethylenediamine | | ,, | 0.8 |
| 277 | 9215 | N: N′-bis-*cyclo*ethylenecarbamyl-*p*-*p*′-diaminodiphenylmethane |  | a.d. | 6.5 |
| 278 | 9608 | N: N′-bis-*cyclo*ethylenecarbamyl-*p*: *p*′-diamino-di-*cyclo*hexylmethane |  | ,, | 1.3 |
| 279 | 9604 | N: N′-bis-*cyclo*ethylenecarbamyl-2: 4-diaminotoluene |  | o.d. | 1.2 |
| 280 | 9606 | N: N′: N″-tris-*cyclo*ethylenecarbamyl-2: 4: 6-triaminotoluene |  | a.d. | 5.0 |
| 281 | 9607 | N: N′-bis-*cyclo*ethylenecarbamyl-2: 4-diaminochlorobenzene |  | o.d. | 8.0 |

TABLE II (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 44.2 | 0.5 | 31.6 | 3.8 | 88 | 1.0 | 45 | Some inhibition of mitosis. A few *D* and *E* | A few *B*, *F*, and *S* |
| 53.7 | 13.2 | 48.5 | 1.3 | 97 | — | — | — | — |
| 35.4 | 8.7 | 37.9 | 12.7 | 67 | — | — | — | — |
| 30.6 | −9.9 | 31.7 | 2.0 | 94 | 0.4 | 88 | | No sample |
| 30.6 | 6.4 | 31.7 | 1.2 | 96 | 0.4 | | Mitosis almost completely inhibited. A few *D* and *E* | *B*, *F*, and *S*, and some *D* and *P* |
| | | | | | 0.1 | 53 | | |
| 22.4 | 13.0 | 28.4 | 7.7 | 73 | 2.5 | | Mitosis almost completely inhibited. Some *B*, *F*, and *E* | No sample |
| | | | | | 1.0 | 68 | A few *D*, *E*, and *M* | |
| 30.6 | 6.8 | 31.7 | 0.5 | 98 | 5 | | Mitosis almost completely inhibited | Divisions few. Polymorphs swollen; many D |

TABLE II (*continued*)

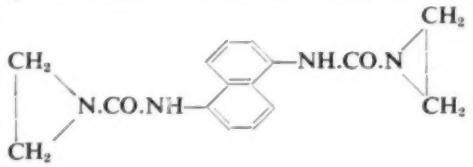
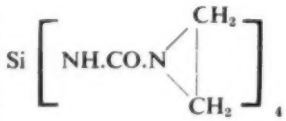
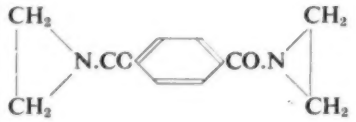
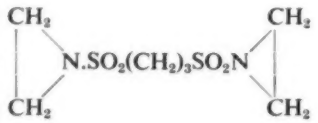
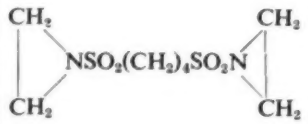
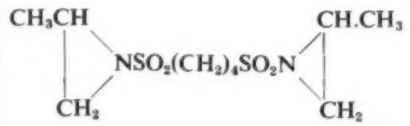
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| 282 | 9860 | N: N′-bis-*cyclo*-ethylenecarbamyl-1 : 5-diamino-naphthalene |  | a.d. | 8.0 |
| 283 | 9815 | Silicon tetra-*cyclo*ethylene urea |  | ,, | 27.5 |
| | | CARBOXYLIC ACID AMIDES | | | |
| 284 | 9959 | Succinylethyleneimine | | a.s. | 3.0 |
| 285 | 9958 | Adipylethyleneimine | | ,, | 5.5 |
| 286 | 9732 | Terephthalylethyleneimine |  | ,, | 11 |
| | | SULPHONAMIDES | | | |
| 287 | 10694 | ω: ω′-bis-(ethyleneiminosulphonyl)-propane |  | a.d. | 0.9 |
| 288 | 10796 | ω: ω′-bis-(ethyleneiminosulphonyl)-butane |  | ,, | 1.8 |
| 289 | 10799 | ω: ω′-bis-(C-methylethyleneiminosulphonyl)butane |  | ,, | 30.0 |

TABLE II (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 33.8 | 9.4 | 39.0 | 5.2 | 87 | 4 | 65 | | No sample |
| 22.0 | 16.2 | 41.8 | 40.1 | 4 | — | — | — | — |
| 22.0 | 14.0 | 41.8 | 44.2 | 0 | 2 | 1–6 | | Normal |
| 20.7 | 13.0 | 34.6 | 20.8 | 40 | 1.0 | 77 | Some inhibition of mitosis; some *D* and *E* | A few *B* and *F* |
| 50.0 | 12.7 | 44.8 | 24.9 | 44 | 10 | 14 | Some inhibition of mitosis | Several *D* and *P*; a few *B* and *F* |
| | | | | | 5 | 13 | | A few *B* and *F* |
| 24.0 | −4.6 | 29.1 | 0.6 | 98 | 0.4 | | Almost complete inhibition of mitosis. A few *F* | Severe damage; no division; many *D* |
| | | | | | 0.2 | 90 | Some *D* and *E* | |
| 32.8 | −11.9 | 37.7 | 0.25 | 99 | 2.0 | | Complete inhibition of mitosis; many *D* and *E* | Severe damage; many *D* and *P* |
| | | | | | 1.0 | 97 | | |
| 32.8 | −8.6 | 37.7 | 0.09 | >99 | 10 | | Almost complete inhibition of mitosis; a few *F* | Many *D* and *E* |

TABLE II (*continued*)

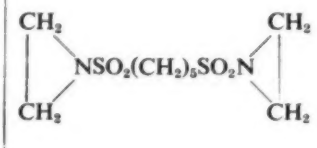
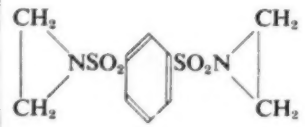
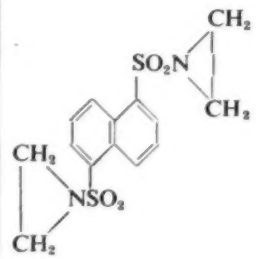
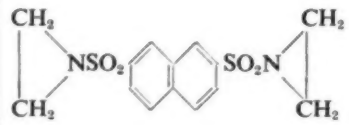
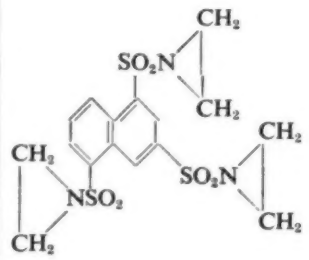
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| 290 | 10695 | ω: ω′-bis-(ethylene-iminosulphonyl)-pentane |  | a.d. | 1.6 |
| 291 | 10797 | ω: ω′-bis-(ethylene-iminosulphonyl)-octane | | ,, | 23 |
| 292 | 9953 | *m*-bis-(ethylene-iminosulphonyl)-benzene |  | ,, | 22.5 |
| 293 | 9814 | 1: 5-bis-(ethylene-iminosulphonyl)-naphthalene |  | ,, | 150 |
| 294 | 9813 | 2: 7-bis-(ethylene-imino sulphonyl)-naphthalene |  | ,, | 80 |
| 295 | 9817 | 1: 3: 5-tris(ethylene-imino sulphonyl)-naphthalene |  | ,, | 125 |

TABLE II (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 24.0 | −7.0 | 29.1 | 0.06 | >99 | 0.5 | | Complete inhibition of mitosis | Severe damage; no divisions, many *D* and *P* |
| | | | | | 0.25 | | Almost complete inhibition of mitosis; some *B* and *F* | |
| 34.6 | −4.4 | 46.1 | 0.9 | 98 | 5 | | Almost complete inhibition of mitosis; a few *F* | Some *D* and *P* and some *B* and *F* |
| 27.5 | 13.4 | 20.9 | 12.7 | 40 | 2.5 | | Complete inhibition of mitosis | Some *B*, *F*, and *D* |
| 16.8 | −3.3 | 27.8 | 3.9 | 86 | 100 | 30 | Some *E* and *M* | A few *F* |
| 16.8 | −11.6 | 27.8 | 2.6 | 91 | 25 | 51 | A few *D* | A few *B* and *F* |
| 16.8 | −3.1 | 27.8 | 11.9 | 57 | 25 | 28 | | ,, |

TABLE II (*continued*)

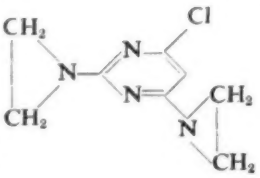
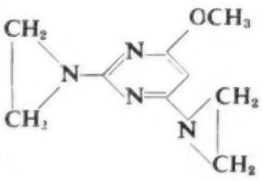
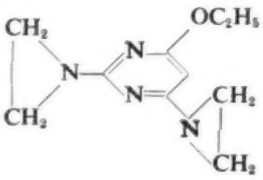
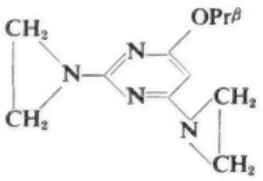
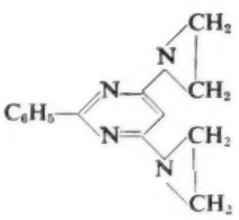
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| | | PYRIMIDINES | | | |
| 296 | 9955 | 4-chloro-2: 6-bis-ethyleneimino-pyrimidine |  | a.s. | 0.7 |
| 297 | 10552 | 2: 4-bis-ethylene-imino-6-methoxy-pyrimidine |  | ,, | 0.55 |
| 298 | 10553 | 2: 4-bis-ethylene-imino-6-ethyoxy-pyrimidine |  | ,, | 0.9 |
| 299 | 10575 | 2: 4-bis-ethylene-imino-6-*iso*propoxy pyrimidine |  | o.s. | 0.45 |
| 300 | 10208 | 4: 6-bis-ethylene-imino-2-phenyl-pyrimidine |  | a.d. | 8.0 |

TABLE II (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | *A* | Remarks | |
| 33.7 | 9.7 | 37.4 | 0.4 | 99 | 0.25 | | Almost complete inhibition of mitosis. Many *E*, some *F* | Many *D*, some *B* and *F* |
| 34.2 | 5.3 | 33.4 | 0.7 | 98 | 0.125 | | Almost complete inhibition of mitosis. Many *D* and *E*; a few *F* | No divisions. Many cells necrotic and degenerating |
| 45.7 | 0.6 | 42.5 | 1.5 | 97 | 0.05 | 63 | Some inhibition of mitosis | A few *B*, *F*, and *S* |
| 45.7 | 6.9 | 42.5 | 2.2 | 95 | 0.125 | | Almost complete inhibition of mitosis; some *E* and *F* | No divisions; many *D* |
| 33.0 | 10.4 | 27.5 | 1.75 | 94 | 1.0 | | Complete inhibition of mitosis | A few *F* and *S* |
| | | | | | 0.5 | 84 | Many *D* and *E* | |

TABLE II (*continued*)

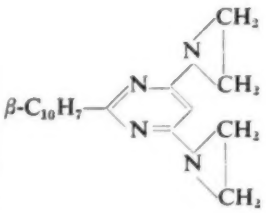
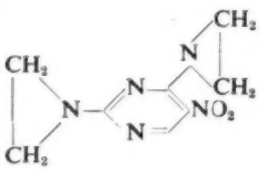
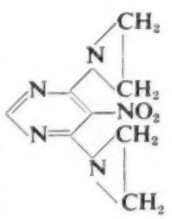
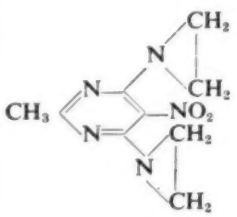
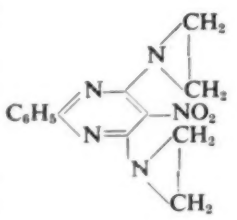
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| 301 | 10404 | 4 : 6-bis-ethylene-imino-2-β-naphthyl pyrimidine |  | a.d. | 2.5 |
| 302 | 9896 | 5-nitro-2 : 4-bis-ethyleneimino-pyrimidine |  | ,, | 2.0 |
| 303 | 9895 | 5-nitro-4 : 6-bis-ethyleneimino-pyrimidine |  | ,, | 20 |
| 304 | 9894 | 5-nitro-4 : 6-bis-ethyleneimino-2-methylpyrimidine |  | ,, | 17 |
| 305 | 10037 | 5-nitro-4 : 6-bis-ethyleneimino-2-phenylpyrimidine |  | ,, | 112.5 |

TABLE II (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 23.2 | 3.9 | 34.3 | 0.64 | 98 | 0.5 | | Complete inhibition of mitosis. Some *D* and *E* | A few *F* and *S* |
| | | | | | 0.25 | 64 | Some *D* and *E* | |
| 31.2 | 12.8 | 39.0 | 2.4 | 94 | 1.0 | | Complete inhibition of mitosis; many *D* | No specimen |
| 45.9 | 20.4 | 42.3 | 3.0 | 93 | 5.0 | | Complete inhibition of mitosis; many *D* | Many *D* and *P* |
| 45.9 | 12.8 | 42.3 | 0.9 | 98 | 2.0 | | Almost complete inhibition of mitosis; some *B* and *F* and many *D* and *E* | Some *B* and *F* |
| | | | | | 1.0 | 87 | Some inhibition of mitosis; some *D*, *E*, and *M* | |
| 37.0 | 11.2 | 40.8 | 19.1 | 53 | 75 | 61 | Some *D* and *E* | A few *B* and *F* |

TABLE II (*continued*)

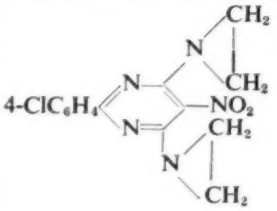
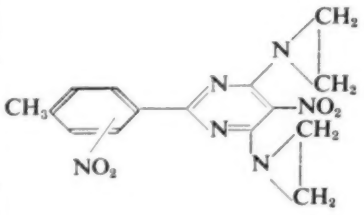
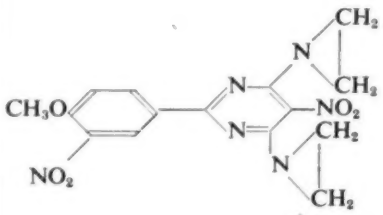
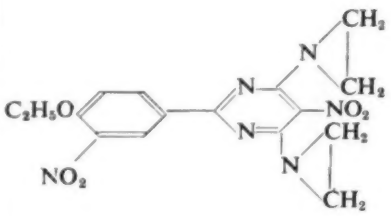
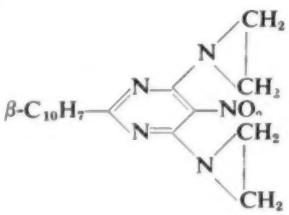
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| 306 | 10406 | 5-nitro-4 : 6-bis-ethyleneimino-2-*p*-chlorophenyl-pyrimidine |  | a.d. | 17.5 |
| 307 | 10550 | x′ : 5-dinitro-4 : 6-bis-ethylene-imino-2-*p*-tolyl-pyrimidine |  | ,, | 10 |
| 308 | 10316 | 3′ : 5-dinitro-4 : 6-bis-ethylene-imino-2-*p*-anisyl-pyrimidine |  | ,, | 27.5 |
| 309 | 10167 | 3′ : 5-dinitro-4 : 6-bis-ethylene-imino-2-*p*-phenetylpyrimidine |  | ,, | 212.5 |
| 310 | 10382 | 5-nitro-4 : 6-bis-ethyleneimino-2-β-naphthyl-pyrimidine |  | ,, | 35 |

TABLE II (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 45.5 | 0.8 | 42.9 | 15.9 | 63 | 20 | 4–7 | | A few *S* |
| 16.6 | 6.1 | 27.4 | 22.5 | 17.9 | 1.0 | 1 | | Normal |
| 16.6 | −2.1 | 27.4 | 18.8 | 31 | 2.5 | 0 | | A few *S* |
| 32.7 | 10.4 | 42.6 | 21.8 | 49 | 100 | 3 | Some inhibition of mitosis | ,, |
| 35.2 | −10.6 | 38.3 | 8.0 | 79 | 100 | 1 | | ,, |

TABLE II (*continued*)

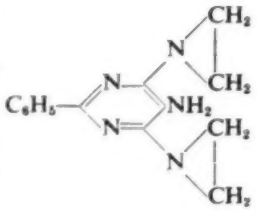
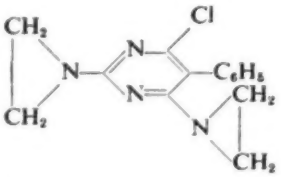
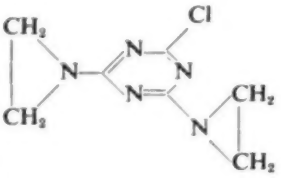
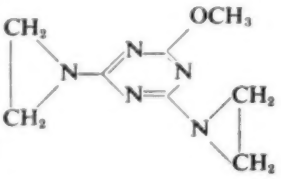
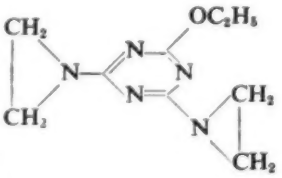
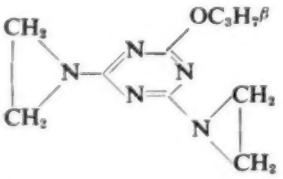
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 g. i.p.) |
|---|---|---|---|---|---|
| 311 | 10665 | 5-amino-4 : 6-bis-ethyleneimino-2-phenylpyrimidine |  | a.d. | 5 |
| 312 | 10426 | 4-chloro-2 : 6-bis-ethyleneimino-5-phenylpyrimidine |  | ,, | 2 |
| | | 1 : 3 : 5-TRIAZINES | | | |
| 313 | 10568 | 2-chloro-4 : 6-bis-ethyleneimino-1 : 3 : 5-triazine |  | o.d. | 1.3 |
| 314 | 10379 | 2 : 4-bis-ethyleneimino-6-methoxy-1 : 3 : 5-triazine |  | a.s. | 1.0 |
| 315 | 10380 | 2 : 4-bis-ethyleneimino-6-ethoxy-1 : 3 : 5-triazine |  | ,, | 1.5 |
| 316 | 10381 | 2 : 4-bis-ethyleneimino-6-*iso*propoxy-1 : 3 : 5-triazine |  | ,, | 2.0 |

TABLE II (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 36.7 | 33.7 | 41.8 | 18.6 | 56 | 0.5 | | Almost complete inhibition of mitosis, many *E* and *D* and some *F* | A few *B*, *F*, and *S* |
| 23.2 | 2.9 | 34.3 | 1.5 | 96 | 0.5 | | Complete inhibition of mitosis; many *E* and *D* | Some *D* and *P* |
| 45.7 | 9.4 | 42.5 | 20.3 | 52 | 0.4 | 56 | | Many *B* and *F* |
| | | | | | 0.2 | 45 | | Some *B* and *F* |
| 35.2 | −0.5 | 38.3 | <0.2 | >99 | 1.0 | | Complete inhibition of mitosis | Many *D* and *P* |
| | | | | | 0.5 | 78 | Some inhibition and some *E* | |
| 16.6 | −6.0 | 30.2 | 0.8 | 97 | 1.0 | | Complete inhibition of mitosis; some *E* | Many *D* and *P*; no divisions |
| | | | | | 0.5 | 98 | Some inhibition | Many *D* and *P* and some *F* |
| 15.0 | 1.6 | 29.1 | 1.1 | 96 | 1.0 | 92 | | Many *D*, *P*, and *E* and some *F* |

TABLE II (*continued*)

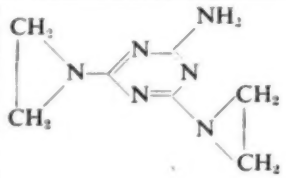
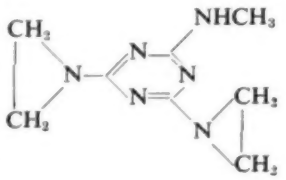
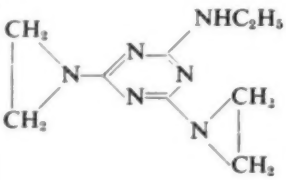
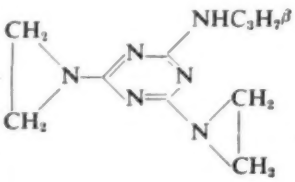
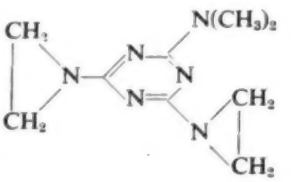
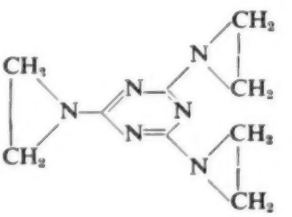
| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./100 i.p.) |
|---|---|---|---|---|---|
| 317 | 9733 | 2-amino-4 : 6-bis-ethyleneimino-1 : 3 : 5-triazine |  | a.d. | 0.18 |
| 318 | 9858 | 2-methylamino-4 : 6-bis-ethyleneimino-1 : 3 : 5-triazine |  | a.s. | 0.4 |
| 319 | 9912 | 2-ethylamino-4 : 6-bis-ethyleneimino-1 : 3 : 5-triazine |  | ,, | 0.35 |
| 320 | 9859 | 2-*iso*propylamino-4 : 6-bis-ethyleneimino-1 : 3 : 5-triazine |  | ,, | 0.4 |
| 321 | 9960 | 2-dimethylamino-4 : 6-bis-ethylene-imino-1 : 3 : 5-triazine |  | ,, | 0.13 |
| 322 | 9500 | 2 : 4 : 6-tris-ethylene-imino-1 : 3 : 5-triazine |  | ,, | 0.11 |

TABLE II (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\angle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 32.4 | 25.9 | 31.6 | 0.7 | 98 | — | — | — | — |
| 28.5 | 11.5 | 37.2 | 1.6 | 96 | 0.01 | | Almost complete inhibition of mitosis. A few *M* | No specimen |
| 28.5 | 20.5 | 37.2 | 1.2 | 97 | — | — | — | — |
| 28.5 | 13.6 | 37.2 | 1.2 | 97 | 0.01 | 74 | A few *M* | No specimen |
| 26.7 | 18.3 | 30.3 | 0.1 | >99 | — | — | — | — |
| 17.0 | 7.0 | 39.6 | 0.7 | 98 | See Table V | | | |

TABLE II (*continued*)

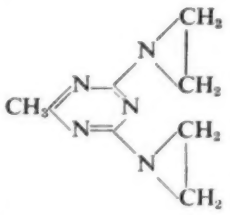
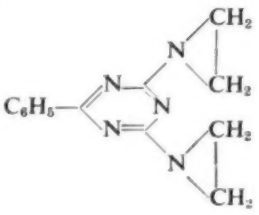
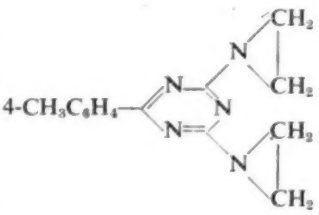
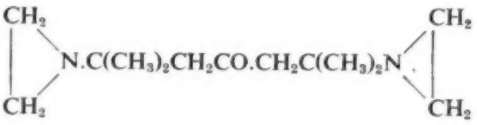
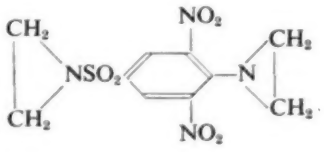
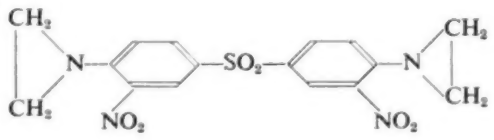
| Serial No. | Code Number | Name | Formula | Form | Total do (mg./10 i.p.) |
|---|---|---|---|---|---|
| 323 | 10306 | 2: 4-bis-ethylene-imino-6-methyl-1: 3: 5-triazine |  | a.s. | 0.6 |
| 324 | 10042 | 2: 4-bis-ethylene-imino-6-phenyl-1: 3: 5-triazine |  | a.d. | 6 |
| 325 | 10305 | 2: 4-bis-ethylene-imino-6-*p*-tolyl-1: 3: 5-triazine |  | ,, | 9 |
| | | MISCELLANEOUS | | | |
| 326 | 9816 | N: N′-di-(β-ethylene-imino-ethyl)-oxamide | | ,, | 0.6 |
| 327 | 10573 | di-(γ-ethyleneimino-propyl)amine | | a.s. | 0.2 |
| 328 | 10549 | β: β′-bis-ethylene-imino-di-*iso*-butyl ketone |  | ,, | 12 |

TABLE II (*continued*)

| Total dose (mg./100 g. i.p.) | Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| | Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 0.6 | 16.6 | 6.7 | 30.2 | 1.5 | 95 | 0.5 | | Almost complete inhibition of mitosis. Many *D* and *E* | Many *D* and *P* and extensive disorganization ; no divisions |
| | | | | | | 0.25 | 91 | | No specimen |
| 6 | 14.0 | 2.5 | 26.5 | 1.1 | 96 | 2.0 | | Almost complete inhibition of mitosis ; many *D* and *E* | Some *D* and *P*; a few *S* |
| | | | | | | 1.0 | 76 | Many *D* and *E* | Some *B*, *F*, and *S* |
| 9 | 38.9 | 9.8 | 39.3 | 2.7 | 93 | 2.0 | | Some inhibition of mitosis ; many *F* and some *E* | Some *B*, *F*, and *S* and *D* and *P* |
| | | | | | | 1.0 | 12 | | A few *S* |
| 0.6 | 16.8 | 16.4 | 27.8 | 6.7 | 76 | 1.0 | 93 | Some inhibition of mitosis ; many D | *B* and *F* and many *P* and *D* |
| 0.2 | 44.1 | 6.8 | 31.6 | 15.8 | 50 | 0.4 | | Complete inhibition of mitosis; many *D* and *E* | Many *D* and *P* |
| | | | | | | 0.2 | 22 | A few *D* and *E* | A few *S* |
| 12 | 44.1 | 4.1 | 31.6 | 17.2 | 46 | 4.0 | | Complete inhibition of mitosis; some *F* and many *D* and *E* | A few *B* and *S* and some *F* |

TABLE II (*continued*)

| Serial No. | Code Number | Name | Formula | Form | Total dose (mg./10 i.p.) |
|---|---|---|---|---|---|
| 329 | 10754 | 2 : 6-dinitro-4-ethyleneimino-sulphonyl-*N*-phenylethyleneimine |  | a.d. | 15 |
| 330 | 10666 | *m* : *m*′-dinitro-*p* : *p*′-bis-ethyleneimino-diphenylsulphone |  | ,, | 125 |
| 331 | 9954 | Polymer from 2-chloro-4-ethylene-iminopyrimidine | | ,, | 110 |
| 332 | 10359 | Polymer from N : N′-bis-*cyclo*ethylene urea | | a.s. | 15 |

be made, but comparison of the bis-ethyleneimino-pyrimidines 296–299 with the corresponding triazines 313–316 shows that the former nucleus consistently provides the more active compound. The introduction of a bulky substituent such as phenyl into either the pyrimidine or triazine rings in these structures markedly reduces activity.

Because of the asymmetry of the pyrimidine nucleus the bis-ethyleneimino compounds derived therefrom are of two isomeric forms, 2 : 4- and 4 : 6- respectively. The latter would appear to be the less active, but in order to prepare them additional substituents (phenyl or nitro) had to be introduced into the nucleus, and this may account, in part at least, for their relatively lower activity.

Activity is high also in some compounds where the ethyleneimine residues are linked by other conjunctive groups. Thus, for example, the bis-urea, 276, derived from 1 : 6-hexamethylene diamine, is almost as effective as tris-ethyleneimino-triazine, while other bis-ureas, including some in which the link is part cyclic and part acyclic, are also strongly inhibitory of tumour growth. Of a small series of related dicarboxylamides, adipyl-ethyleneimine, 285, and terephthalyl-ethyleneimine, 286, are slightly active, while succinyl-ethyleneimine, 284, produces no inhibition in maximal tolerated doses. The low activity of this class is possibly due to

TABLE II (*continued*)

| Tumour inhibition | | | | | Cytotoxic action | | | |
|---|---|---|---|---|---|---|---|---|
| $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per 100 g. i.p.) | Tumour | | Bone marrow |
| Control | Treated | Control | Treated | | | $A$ | Remarks | |
| 48.2 | 11.7 | 38.4 | 4.8 | 88 | 20 | | Complete inhibition of mitosis; many *D* | *D* and *P*; also some *B*, *F*, and *S* |
| | | | | | 10 | 71 | — | As above, but not so marked |
| 31.6 | −1.7 | 40.4 | 1.4 | 97 | 40 | | Complete inhibition of mitosis | A few *S* |
| | | | | | 10 | 12 | | ,, |
| 14.0 | 18.0 | 26.5 | 30.7 | 0 | 20 | 6 | | Normal |
| 39.7 | 28.9 | 49.4 | 40.3 | 18 | 1.0 | 14 | | ,, |

secondary intramolecular condensations leading to unreactive oxazoline derivatives. With the sulphonamides, 287–295, where such auto-condensation cannot occur, higher activity is found. With aliphatic derivatives such as $\omega : \omega'$-bis-(ethyleneimino-sulphonyl) propane, 287, total doses of the order of 1 mg. per 100 g. are strikingly effective, whereas in the bis- and tris-sulphonamido derivatives of naphthalene, e.g., compounds 293–295, the maximum tolerated doses are of the order of one hundred times greater, and even with these large doses full tumour inhibition is difficult to achieve. It is suggested that this result is attributable to the lower solubility and consequent poorer absorption of the latter substances. It will be noted that considerably larger doses of the bis-C-methylethyleneimine 289 than of the unmethylated 288 are tolerated, but that with a comparable effect upon tumour growth.

Table III contains miscellaneous compounds related to active and inactive ethyleneimines and examined in that connexion. The two trimethyleneimine derivatives, 333 and 334, corresponding to active ethyleneimines, have little action in high doses, as indeed might be expected from the relatively greater stability of the four membered ring. The four compounds following, 335–338, are related to the active dinitrophenyl-ethyleneimine 257, from which they differ in having in

TABLE III

ACTION ON THE WALKER TUMOUR OF MISCELLANEOUS COMPOUNDS RELATED TO THE ETHYLENEIMINES

Conventions as in Tables I and II

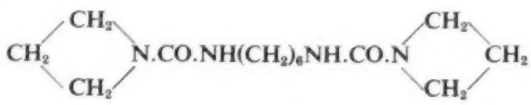
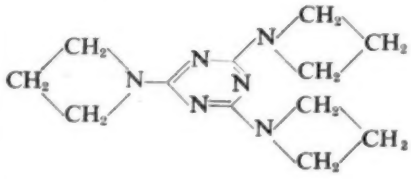
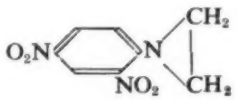
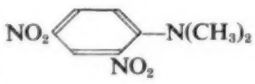
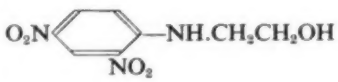
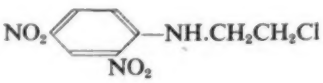
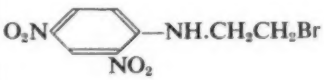
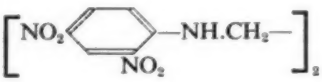
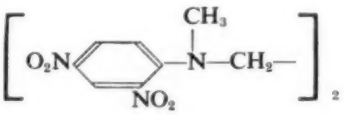
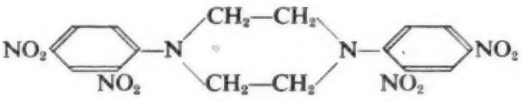
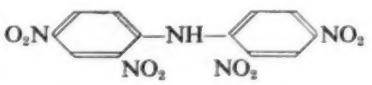
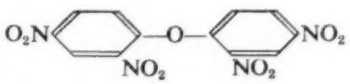
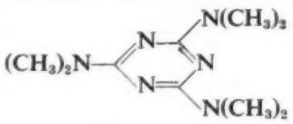
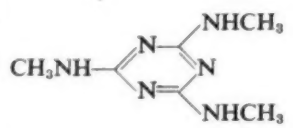
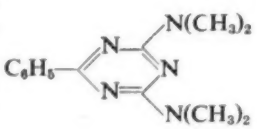
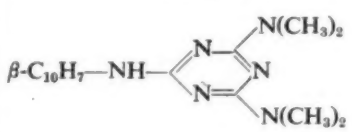
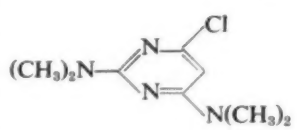
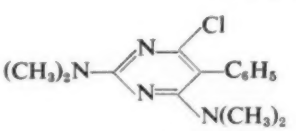
| Serial No. | Code Number | Name | Formula | Form |
|---|---|---|---|---|
| | | TRIMETHYLENEIMINES | | |
| 333 | 10202 | N: N′-bis-*cyclo*-trimethylene carbamyl hexamethylene diamine |  | a.d. |
| 334 | 10071 | 2: 4: 6-tris-trimethyleneimino-1: 3: 5-triazine |  | ,, |
| | | COMPOUNDS BEARING ON ACTIVITY OF |  | |
| 335 | 10424 | 2: 4-dinitrophenyl-dimethylamine |  | ,, |
| 336 | 10560 | N-2: 4-dinitrophenyl-ethanolamine |  | ,, |
| 337 | 10561 | N-β-chloroethyl-2: 4-dinitroaniline |  | ,, |
| 338 | 10562 | N-β-bromoethyl-2: 4-dinitroaniline |  | ,, |
| 339 | 10677 | N: N′-bis-(2: 4-dinitrophenyl)-ethylene-diamine |  | ,, |
| 340 | 10676 | N: N′-dimethyl-N: N′-bis-(2: 4-dinitrophenyl) ethylene-diamine |  | ,, |
| 341 | 10675 | N: N′-bis-(2: 4-dinitro-phenyl)-piperazine |  | ,, |

TABLE III (*continued*)

| Total dose (mg./100 g. i.p.) | Tumour inhibition | | | | | Cytotoxic action | | |
|---|---|---|---|---|---|---|---|---|
| | $\triangle W$ | | $M_{50}$ | | *I* | Dose (mg. per 100 g. i.p.) | Tumour | Bone marrow |
| | Control | Treated | Control | Treated | | | *A* | |
| 200 | 32.7 | 33.3 | 42.6 | 38.9 | 9 L | — | — | — |
| 75 | 32.7 | 27.4 | 42.6 | 30.0 | 30 L | 40 | 4 | Normal |
| 120 | 24.6 | 8.4 | 23.0 | 25.0 | 0 | 40 | 2 | ,, |
| 160 | 25.0 | 16.8 | 24.5 | 22.1 | 10 | 10 | 1 | A few *S* |
| 60 | 31.6 | 27.2 | 40.4 | 37.0 | 8 | 10 | 1 | ,, |
| 30 | 32.8 | 24.7 | 37.8 | 34.5 | 9 L | 20 | 0 | ,, |
| 90 | 34.2 | 21.9 | 33.4 | 20.9 | 37 | 12 | 0 | Normal |
| 150 | 36.7 | 37.6 | 41.8 | 38.3 | 8 L | 75 | 0 | ,, |
| 75 | 36.7 | 31.1 | 41.8 | 34.7 | 17 L | 40 | 0 | ,, |

TABLE III (*continued*)

| Serial No. | Code Number | Name | Formula | Form |
|---|---|---|---|---|
| 342 | 10753 | bis-(2 : 4-dinitrophenyl)amine |  | a.d. |
| 343 | 10793 | bis-(2 : 4-dinitrophenyl) ether |  | ,, |
| | | COMPOUNDS BEARING ON ACTIVITY OF HEXAMETHYL MELAMINE | | |
| 344 | 10567 | 2 : 4 : 6-tris-dimethylamino-1 : 3 : 5-triazine |  | ,, |
| 345 | 8758 | 2 : 4 : 6-tris-methylamino-1 : 3 : 5-triazine |  | a.s. |
| 346 | 10953 | 2 : 4-bis-dimethylamino-6-phenyl-1 : 3 : 5-triazine |  | a.d. |
| 347 | 10954 | 2 : 4-bis-dimethylamino-6-β-naphthylamino-1 : 3 : 5-triazine |  | ,, |
| 348 | 10794 | 4-chloro-2 : 6-bis-dimethylamino-pyrimidine |  | ,, |
| 349 | 10795 | 4-chloro-2 : 6-bis-dimethylamino-5-phenyl-pyrimidine |  | ,, |

place of the ethyleneimine residue a grouping which might be expected to behave in an analogous manner. All four compounds were inactive. In the next five, 339–343, two 2 : 4-dinitrophenyl residues are linked together by various conjunctive groups. These too were inactive.

TABLE III (*continued*)

| Total dose (mg./100 g. i.p.) | Tumour inhibition | | | | | Cytotoxic action | | |
|---|---|---|---|---|---|---|---|---|
| | $\triangle W$ | | $M_{50}$ | | $I$ | Dose (mg. per i.p.) | Tumour | Bone marrow |
| | Control | Treated | Control | Treated | | | $A$ | |
| 97.5 | 33.0 | 31.2 | 30.5 | 40.2 | 0 | 30 | 2 | A few *S* |
| 65 | 34.6 | 16.1 | 46.1 | 37.8 | 18 | 20 | 0 | Normal |
| 80 | 25.0 | −5.4 | 24.5 | 4.8 | 79 | 10 | 31 | A few *S* and *P* |
| 147.5 | 32.8 | 12.4 | 37.7 | 14.9 | 60 | 20 | 18 | A few *F* and *S* |
| 205 | 27.4 | 7.5 | 32.9 | 19.7 | 40 L | 50 | 7 | Normal |
| 220 | 27.4 | 2.3 | 32.9 | 21.4 | 35 | 50 | 0–7 | ,, |
| 67.5 | 34.6 | 25.7 | 46.1 | 36.1 | 22 | 10 | 0 | ,, |
| 300 | 34.6 | 10.1 | 46.1 | 24.1 | 48 | 50 | 0 | ,, |

The demonstration that tris-(dimethylamino)-triazine, 344, is active confirms a finding of Buckley, Stock, Crossley, and Rhoads (1950) that the substance produces slight but definite inhibition of the Crocker mouse sarcoma 180. This result is considered further in the general discussion.

*Cytotoxic action* (with J. M. Gates)

The majority of the compounds listed in Tables I to III have been examined for the capacity to induce chromosome effects of the type regarded as characteristic of the action of true "radiomimetic" agents in dividing cells of the Walker tumour. Their effect upon the bone marrow of rats was studied at the same time. Single intraperitoneal doses of the compounds were given to rats bearing tumour implants of several days' growth and Feulgen-stained squash preparations were made from tumour tissue and bone marrow taken when the animals were killed 24 hours later. The distinguishing features of these specific chromosome effects have already been described (Hendry, Rose, and Walpole, 1951). They consist essentially of chromosome fragmentation, accompanied by the reunion of fragments in various ways and to a variable extent and leading to the appearance, among other manifestations, of dicentric chromosome bridges and of chromosome fragments at anaphase. These effects are to be distinguished from non-specific "toxic" changes, e.g., chromosome "stickiness" and its sequelae, nuclear pyknosis, etc. The increase over controls in the percentage of anaphases showing abnormalities of the former type in tumour tissue from rats treated with the compounds in the doses shown is recorded in the column headed *A* in Tables I to III, with supplementary remarks in the next column. Qualitative observations upon cytotoxic effects in the bone marrow are included in the same Tables.

It will be seen that a high proportion of the compounds examined in this way caused pronounced cytotoxic effects in the tumour cells. Typical of the more active in this respect—for details with one such, 322, see Table V—was the production, with comparatively low doses, of specific chromosome damage and in addition, with higher doses, an inhibition of mitosis which might be complete. A full range of doses was not always examined and both these distinct but presumably related phenomena were not always observed. Thus with some few compounds, e.g., 302 and 303, complete mitotic inhibition only was seen with the doses tested, but although this action is not restricted to "radiomimetic" agents the general picture here is such that it seems reasonable to assume that lower doses of the compounds in question would have caused the appearance of specific chromosome effects. The converse, however, appears not to be true in that with some compounds, e.g., 282, the highest doses which could be given failed to produce marked inhibition of mitosis.

The above compounds as a whole, where *A* is greater than 15 per cent, were active inhibitors of tumour growth. To this generalization only five exceptions have been found, namely, 258, 261, 292, 327, 328. It should be pointed out, however, that the doses which can be given in an acute (24-hour) test for cytotoxic activity often greatly exceed those tolerated in chronic experiments upon the inhibition of tumour growth, where some toxic action of a compound unrelated to its specific effect upon the chromosomes may be the factor limiting dosage. This may account for the failure to obtain pronounced tumour inhibition with these few compounds.

With those compounds which appeared completely devoid of specific cytotoxic activity or with which the proportion of anaphases in treated tumours showing

specific chromosome effects was small (*A* less than 15 per cent) tumour-inhibitory activity was, in general, negligible. With some few of these compounds, e.g., 268, the inhibition of tumour growth appeared to be rather greater than could be accounted for by non-specific "toxicity" as judged by their effect on gross weight gain (Walpole, 1951), but with none of these compounds did it exceed 66 per cent.

*Carcinogenic activity*

Of the compounds listed in Tables I, II, and III, only two, namely, stearoyl-ethyleneimine, 248, and tris-ethyleneimino-1 : 3 : 5-triazine, 322, have been examined in such a way that carcinogenic activity, if present, might be detected. The results with the former are described below, and those with the latter in the section (page 398) devoted to that compound. Other ethyleneimine derivatives are on test in connexion with theories developed in the Discussion (page 408).

*Stearoyl-ethyleneimine, 248*

A 2 per cent solution of the compound in arachis oil was injected subcutaneously in the right flank of stock albino rats, about 100 g. in body weight. Five male and five female rats were each given 10 mg. per 100 g. twice weekly for five weeks, while a similar group were given 10 doses of arachis oil alone (0.5 ml. per 100 g.) and served as controls. Twelve (calendar) months after the first injection no tumour had developed among the controls. In the rats given stearoyl-ethyleneimine progressively growing tumours appeared at the injection site in three of the males and two of the females. Four of these were diagnosed histologically as mixed-cell sarcomata. The first of these appeared between three and four months from the beginning of the experiment, and when the animal was killed at four months was a roughly spherical mass some 2 in. in diameter. The other three were first noted at about six, six, and seven months respectively. Two were successfully transplanted into stock rats; transplantation of the other two was not attempted. The fifth subcutaneous tumour, which appeared at about eight months, was a spindle-cell sarcoma. One other of the females lost condition rapidly at about 11 months and was killed. A mixed-cell sarcoma was found filling the thoracic cavity and infiltrating the heart muscle and lung.

The stearic acid from which the stearoyl-ethyleneimine used in this experiment was prepared was the commercial product and undoubtedly contained some unsaturated material together with higher and lower homologues. The experiment is now being repeated with a product obtained from specially purified stearic acid containing only traces of other acids.

*Tris-ethyleneimino-1 : 3 : 5-triazine, 322*

On account of the profound inhibition of the growth of the Walker tumour produced by this substance in minute doses its properties have been studied in detail with results which are summarized below. These findings in no way contradict those already published by workers at the Sloan-Kettering Institute (see, e.g., Buckley, Stock, Crossley, and Rhoads, 1950; Burchenal, Johnston, Stock, Crossley, and Rhoads, 1950).

The compound is a colourless crystalline solid, readily soluble in water and in many organic solvents. It is stable at low temperatures but readily polymerizes

on being heated. Aqueous solutions have been kept for some months at 4° C. without appreciable change in composition.

*Action on Tumour.*—The inhibition of growth of the Walker tumour produced when it is given by various routes is shown in Table IV. In these experiments it was administered in approximately equal doses daily or on alternate days for the first 10 to 12 days after implantation of the tumour. The percentage inhibition of tumour growth, *I*, was calculated as usual from the tumour weights at 14 days. Deaths occurring among the treated animals before the end of experiment are recorded.

It will be seen that the compound is active by mouth as well as parenterally, but that 50 to 100 times the intravenous dose is required by mouth to produce a comparable effect.

TABLE IV

Effect of route of administration and dosage of tris-ethyleneimino-1 : 3 : 5-triazine (322) upon its growth-inhibitory action on the Walker tumour. The compound was given, except where otherwise indicated, over the first ten to twelve days after implantation of the tumour

| Route of administration | Total dose mg./100 g. rat | *I* (% inhibition of tumour growth) | Rats dead by the 14th day |
|---|---|---|---|
| Intraperitoneal .. .. | 0.11 | 98 | 0/11 |
| ,, .. .. | 0.25 | 99 | 2/22 |
| ,, .. .. | 0.2 (0.1 on days 1 and 2) | 96 | 5/11 |
| Intravenous .. .. .. | 0.055 | 83 | 0/11 |
| ,, .. .. .. | 0.11 | 96 | 0/11 |
| Oral .. .. .. .. | 0.5 | 18 | 0/11 |
| ,, .. .. .. .. | 2.5 | 84 | 1/11 |
| ,, .. .. .. .. | 5.0 | 93 | 0/11 |

In experiments in which tumour inhibition was pronounced it was accompanied by a fairly marked reduction in the increase in gross weight of the treated tumour-bearing rats as compared with that of untreated controls. If dosing was delayed until the implants had been growing for several days, inhibition of their growth was less pronounced and in these circumstances no actual regressions were obtained.

Single intraperitoneal doses given to rats bearing Walker tumours implants of several days' growth produce cytotoxic changes in the tumour cells which vary with the dose. Some results of experiments of this kind are summarized in Table V. Apart from specific "radiomimetic" effects, inhibition of mitosis, also a feature though not a distinguishing feature of "radiomimetic" cell poisons, was observed with doses down to 0.025 mg. per 100 g. Qualitatively similar changes were seen in the cells of the crypts of Lieberkühn in the gut of these animals, but owing to technical difficulties a quantitative comparison was not carried out. In this tissue also mitotic inhibition was in evidence 24 hours after doses down to 0.025 mg. per 100 g., and for 45 hours after doses down to 0.05 mg. Similar changes were seen also in the bone marrow. With higher doses there was an obvious reduction in the cellularity of the marrow, which to the naked eye was a dark red viscous fluid. Degenerating and pyknotic cells were abundant and the

TABLE V

Details of the cytotoxic action of tris-ethyleneimino-1 : 3 : 5-triazine (322) upon the Walker tumour in rats. *B*, true chromosome bridges; *F*, chromosome fragments

| Dose (mg./ 100 g. rat i.p.) | Time after dosing (hr.) | % Anaphases abnormal | | Other effects |
|---|---|---|---|---|
| | | *B* | *F* | |
| 0 | — | 1–3 | 1–2 | ($B + F = 2 — 4$) |
| 0.0125 | 24 | 5–16 | 75–78 | Some " sticky " bridges; some degenerating cells. Some cells with shattered chromosomes, and some with accessory micronuclei |
| | 48 | 10–20 | 55–60 | Many degenerating cells, " exploded " nuclei and cells with micronuclei |
| | 72 | 16 | 40 | As at 48 hours |
| 0.025 | 24 | 16 | 66 | Perhaps some slight inhibition of mitosis |
| | 48 | | | Most cells shattered, the few anaphases visible have chromosome bridges or fragments |
| | 72 | | | As at 48 hours |
| 0.05 | 24 | | | Some inhibition of mitosis; all anaphases abnormal; some degenerating cells |
| | 48 | | | As at 24 hours |
| | 72 | | | Slight inhibition of mitosis. Some normal, some shattered divisions; many degenerating cells |
| 0.1 | 24 | | | Almost complete inhibition of mitosis; all divisions abnormal |
| | 48 | | | Mitosis still largely inhibited; many degenerating cells and cells with accessory micronuclei |
| | 72 | | | No specimen |

megakaryocytes were enormously swollen. With doses in the range covered in Table V, the effects were more comparable with those seen in the tumour and gut.

The specific cytotoxic action of the compound is not limited to mammalian tissues, but as with the agents studied by Loveless and Revell (1949) is seen also, for example, in the dividing cells of the root tips of the broad bean. Growing roots were immersed for an hour in aqueous solutions of 322 at various concentrations and then replaced in water. The tips of lateral roots were taken at intervals thereafter and from them Feulgen-stained squash preparations were made. Vicia chromosomes being fewer in number and much larger than those in the rat, changes in them are much more easily seen and characterized. The results of one such series of observations are shown in Table VI.

*Toxicology of 322*

The toxicology of the compound has been studied in several animal species with results which may be summarized as follows.

*Mice.*—Male stock albino mice, about 20 g. in body weight, were given single intraperitoneal doses of the compound, freshly dissolved in distilled water. All the mice in groups of six given doses of 1,000, 500, 250, and 100 mg. per kg. respectively died within 24 hours, of 50 mg. within three days, and of 25, 10, and 5 mg. within six days. With doses down to 500 mg. per kg. the mice suffered from convulsions ; an attack could be precipitated by sound or mechanical stimulus. On smaller doses they displayed ruffled fur, weakness, splayed hind limbs, coarse tremor, and diarrhoea. The median lethal dose for these mice was 2.5–5 mg. per kg., deaths being recorded up to seven days.

TABLE VI

The cytotoxic action of tris-ethyleneimino-1 : 3 : 5-triazine (322) upon the root tip of the broad bean (Vicia). The growing bean roots were immersed for one hour in aqueous solutions of the compound in the concentrations shown and sampled at the times shown thereafter

| Concentration | Time (hr.) | Appearances |
|---|---|---|
| $10^{-3}$ | 24 | Complete inhibition of mitosis. Nuclear pyknosis widespread and many nuclei darkly stained and coarsely granular appearance (non-specific " toxic " changes) |
| | 48 | Similar to above |
| | 72 | Mitosis still suppressed, but a few prophase nuclei visible. " Toxic " changes in a few cells |
| $10^{-4}$ | 24 | Almost complete inhibition of mitosis with " toxic " changes in some cells |
| | 48 | Divisions few in number. Breaks in some metaphase chromosomes; micronuclei, some multiple, in many cells; slight " toxic " changes |
| | 72 | Divisions more numerous and many normal. Breaks in some metaphase chromosomes; chromosome fragments, numerous in some cells, at anaphase; micronuclei in a few cells |
| $10^{-5}$ | 24 | Marked inhibition of mitosis, a few prophase nuclei being the only evidence of that process. No " toxic " changes |
| | 48 | Divisions still few in number but all stages visible, prophases being the most numerous. Some breaks, both single and double, in metaphase chromosomes; fragments at anaphase. One or more micronuclei in many cells; some nuclei abnormal in shape. |
| | 72 | Mitotic frequency virtually normal. Breaks in some metaphase chromosomes; fragments and a few chromosome bridges at anaphase. Micronuclei in many cells |
| In a second preparation made from a root tip taken twenty-four hours after treatment with the compound at this concentration ($10^{-5}$), chromosome breaks and fragments and/or bridges were seen in about half the dividing cells. | | |
| $10^{-6}$ | 24 | No signs of " toxic " changes. Breaks, both single and double, in metaphase chromosomes with evidence of reconstitution in some cases, one triradiate chromosome being found. Fragments but no bridges at anaphase. Many normal divisions |
| | 48 | Mitotic frequency virtually normal. Some breaks in metaphase chromosomes; some fragments at anaphase; micronuclei in a few cells. Many normal divisions |
| | 72 | As at 48 hours but with a few chromosome bridges at anaphase |

Ten mice dosed once daily for 12 days with intraperitoneal injections of 0.5 mg. 322 per kg. lost weight and died between the tenth and nineteenth days. In a similar group similarly dosed with 0.25 mg. per kg. one died on the tenth and one on the seventeenth day, while the remainder showed some retardation of growth.

Single intraperitoneal doses of 25 mg. per kg. or more produced rapid and widespread damage in the lymphoid tissues and the intestinal mucosa. The blood and bone marrow were not studied in this species. In the spleen of mice killed 24 to 48 hours after being dosed the changes ranged from nuclear fragmentation and marked depletion to complete destruction of the lymphoid cells in both the pulp and the Malpighian corpuscles, with replacement by proliferated reticulo-endothelial cells. In the lymph nodes pyknosis and degeneration of the lymphoid cells were seen. Cessation of mitosis was evident in the crypts of Lieberkühn in the intestine and many degenerating cells were seen there. The liver showed slight vacuolation and a few scattered necrotic cells.

With repeated dosing more extensive and varied changes were produced. The following were noted in addition to those of the types mentioned above: areas of necrosis in the liver and foci of necrosis in the adrenal cortex, in the fibro-fatty tissue associated with the pancreas, and in the glandular tissue of the thyroid. Changes in the tubules of the testes were most striking and were consistently observed ; they varied in severity and included nuclear pyknosis and cellular desquamation, the development of multinucleate giant cells, and loss of spermatogenesis.

*Rats.*—In stock rats of both sexes and about 100 g. in body weight the median lethal dose of the compound by single intraperitoneal injection was 1.5–2 mg. per kg., deaths being recorded up to seven days.

In this species many of the pathological changes were indistinguishable from those seen in mice and as in the latter varied with the level and duration of dosage and the time elapsing before the animals died or were killed. By the third day after a single intraperitoneal injection of 5 mg. per kg. all three rats were moribund, with marked diarrhoea. When killed at this time counts on the heart blood showed a slight elevation of the erythrocyte, and a fall, almost to zero, in the total leucocyte count. The bone marrow was fluid and dark red in colour and in Feulgen-stained smears only a few normal cells could be seen. Pyknotic and degenerating cells were very numerous and the megakaryocytes were swollen. The blood vessels were stuffed with red corpuscles—a change presumably due to haemoconcentration. In the testes the effects seen varied from loss of spermatogenesis with cell desquamation in a few tubules only to complete atrophy. The spleen and abdominal lymph nodes showed acute depletion of lymphoid elements and proliferation of reticulo-endothelial cells. In the adrenal medulla abnormal vacuolation and a few necrotic cells were seen.

*Guinea-pigs.*—Guinea-pigs survived the intraperitoneal injection of 0.1 mg. of 322 per kg. twice daily (Saturdays and Sundays excepted) for three weeks, but during treatment the mean weight of ten pigs fell from 586 to 571 g. In guinea-pigs given a single intraperitoneal dose of 1 mg. per kg. the total leucocyte count fell rapidly, reaching a minimum in about four days. Both polymorphs and lymphocytes were affected.

*Cats.*—The compound was given intravenously in acute experiments with chloralosed cats. In one a dose of 1 mg. per kg. caused no alteration in the blood pressure or respiration ; in another the injection of 10 mg. per kg. produced apnoea and a fall in blood pressure, but when artificial respiration was applied spontaneous breathing restarted and the blood pressure returned to normal levels. In both animals the total leucocyte count fell to a fraction of its original value during the first hour or two and then rose steadily during the next three to four hours to a figure well above the original. At this point the animals were killed.

*Rabbits.*—In rabbits given a single intravenous dose of 1 mg. per kg. the behaviour of the leucocyte count during the first three hours was similar to that in cats. Thereafter the total count fell, reaching a minimum in three to four days and then returned to normal over the next 20 to 30 days. (The fall in three rabbits was from an initial mean value of 6,900 leucocytes per cu.mm. to a mean of 700.) With this treatment the initial rise during the first few hours was due to a polymorphonuclear leucocytosis ; in the subsequent fall to subnormal levels both polymorphs and lymphocytes were affected. The erythrocyte count remained substantially normal throughout.

*Dogs.*—Intravenous doses of 0.05 mg. per kg. were given on successive days to three dogs up to totals of 0.35, 0.45, and 0.45 mg. respectively. The dogs appeared to be quite unaffected by this treatment, but examination of the blood revealed a marked fall in the leucocyte count which reached a minimum a few days after the last dose and returned to normal values over 20 to 30 days. The lymphocytes appeared to be affected sooner and to recover more rapidly than did the polymorphs. The erythrocyte count

did not fall below normal limits. The dogs were killed on days 43, 30, and 30 respectively. The most marked pathological change was that seen in the testis of the one mature male animal, where complete loss of spermatogenesis was evident with cell desquamation, pyknosis, and the development of small multinucleate giant cells. Other changes included cell desquamation and casts in the renal tubules, some congestion of the alveolar capillaries with haemorrhage into the alveoli in the lungs, congestion of the vessels of the duodenal mucosa with denudation of the surface epithelium of the villi, slight vacuolation of parenchymal cells in the liver, and foci of degeneration with polymorph infiltration in the lymphoid tissues.

*Mutagenic activity of 322* (with M. C. Frank)

The compound was tested for mutagenic activity upon spores of the mould *Penicillium chrysogenum*, by the method described in Part II. It was added in concentrations of 0.3 and 0.7 per cent to suspensions containing 3 to $6 \times 10^6$ spores per ml. Samples were taken at intervals between 10 and 90 minutes thereafter, plated on agar medium, and the plates incubated at 24° C. The colonies which developed within a few days were examined and mutants detected by the colour and habit of growth. The results were as follows:

| Concentration of compound | Percentage of mutant colonies produced by 10–90 minutes' exposure | | |
|---|---|---|---|
| % | Exp. *a* | Exp. *b* | Mean |
| 0.7 | 11.7 | 8.0 | 9.85 |
| 0.3 | | 2.7 | |
| 0.0 | 3.6 | 1.5 | 2.55 |

The effect of the higher concentration only is significant. In general the mutants grew more slowly and produced less penicillin than the culture from which they arose.

*Carcinogenic activity of 322*

Ten male and ten female stock albino mice were given the compound subcutaneously once a week in a dose of 0.025 mg. per 20 g. Dosing continued until the animals died or were killed. Ten died during the first twelve months and the remainder were killed about a month later. No signs of malignant disease were found in any of these mice.

Ten male strain A mice, three months old, were given ten intraperitoneal injections of 0.0075 mg. of the compound on alternate days and killed 100 days after the first dose. Pulmonary adenomata were found in eight of the mice, the number in individual animals being 5, 2, 2, 2, 2, 1, 1, 1. In an untreated control group of 15 animals killed at the same age, one had three adenomata in the lungs and one other only one. Essentially similar results have been obtained by Shimkin (1951) and are taken as evidence for low-grade carcinogenic activity.

*Clinical trials of 322*

Clinical trial of the compound in human malignant disease is in progress at various centres in this country and America. Preliminary reports were presented at the Fifth International Cancer Congress in Paris in July, 1950 (to appear in the Unio Internationalis Contro Cancrum Acta), and it is understood that more detailed papers are now in the press (see Note (1) added in proof, page 410).

## Discussion

### *Tumour inhibition*

From the results with ethyleneimine derivatives presented above and from the essentially similar findings with poly-methylolamides and bis-epoxides recorded in Parts I and II, it is not unreasonable to conclude that tumour inhibition with these substances (as well as with *x* rays and the sulphur and nitrogen mustards) results from their cytotoxic action, which appears to be associated with primary changes in the chromosomes and is directed preferentially, if not exclusively, towards dividing cells. This action is not restricted to malignant cells but extends to proliferating cells of all types. It is unnecessary, however, to postulate any special affinity of these compounds for malignant cells to account for the inhibition of tumour growth observed in our experiments. It may well be that the damage initially sustained by mitotically active cells of normal and malignant tissues is quantitatively as well as qualitatively identical and that the apparent preferential effect upon tumour growth as compared with body growth results from a difference in the subsequent process of proliferative repair. In this connexion it is noteworthy that once the tumour implants have become established and developed a blood supply comparable with that of normal tissues it is more difficult to inhibit tumour growth with sub-lethal doses of these agents. Karnovsky and his collaborators (1947), studying the effects of the standard nitrogen mustards upon tumours in mice and in tissue culture, could find nothing to suggest that these substances had a substantially selective effect upon neoplastic cells. We have compared the action of a single dose of trimethylol melamine upon mitotic activity in the tumour tissue and in the crypts of Lieberkühn in rats bearing the Walker tumour. In both tissues the mitotic frequency fell to a minimum value, at 12 hours, of about one half of its original value, but thereafter recovery was rather more rapid in the gut than in the tumour. Again, Elson (1949), from his study of the effect of *x* rays and of some radiomimetic bis-epoxides upon the Walker tumour, inclines to the opinion that these agents have little if any *selective* inhibitory action upon tumour growth, that their main action is one of growth inhibition both of the animal and of the tumour, but that in favourable circumstances the animal can recover from this toxic action more readily than can the tumour.

Thus it would appear that the special merit of "radiomimetic" agents is that their action is limited in the first instance to the chromosomes of actively dividing cells, as a result of which the mitotic sequence of such cells can be interrupted and lethal chromosomal aberrations induced with the minimum coincidental disturbance of structure or function in fully differentiated and non-dividing cells. Their growth-inhibitory action is therefore accompanied by a minimum of toxic side-effects.

On the other hand, there is evidence that the response in different tissues to a radiomimetic agent does not always run parallel with the mitotic activity of their constituent cells. Thus intense nuclear pyknosis and degeneration such as affects the lymphoid cells throughout the body after the administration of these agents are not seen, for example, in the parenchymal cells of the liver, even when that organ is stimulated to increased proliferative activity by partial hepatectomy. This has been demonstrated by Landing, Seed, and Banfield (1949) in the case of HN2 and by ourselves for trisethyleneimino-1 : 3 : 5-triazine, 322 (unpublished). But this

rapid and widespread destruction of lymphoid elements can scarcely be accounted for by damage to dividing cells only, and here attack upon cells at all stages is almost certainly involved. It is probably more than coincidental that such pronounced lymphotoxic activity is common to many radiomimetic agents, but the reason for it is not yet clear.

*Mutagenic and carcinogenic activity*

The cytotoxic action of "radiomimetic" agents, to which has been attributed the inhibition of tumour and body growth which they produce, involves, as has been seen, permanent structural changes in the chromosomes. Since permanent modifications in chromosome structure of one kind or another in fact constitute the material basis of mutation it is not surprising that mutagenic activity has been encountered with agents of this kind. In seeking to explain their cytotoxic action we postulate a chemical interaction between these substances and cell components of a protein or nucleoprotein nature, which are probably the chromosomes themselves. The requirements, in terms of reactivity and structure, for specific cytotoxic activity are discussed in detail below. The requirements for mutagenic activity are likely to be similar but not identical with them. Since the chromosome changes involved in mutation may be slight, for example, in gene or "point" mutations, whereas for growth-inhibitory effects more drastic damage to chromosome structure and, at least, a preponderance of lethal mutations is required, it is to be expected that in this respect the demands for tumour-inhibitory action will be more exacting than for mutagenicity. This conception finds support in the fact, for example, that simple monofunctional epoxides (Rapoport, 1948) and chloroethylamines (Stevens and Mylroie, 1950 ; Auerbach and Moser, 1950 ; Jensen, Kirk, and Westergaard, 1950), which are certainly not tumour inhibitors, have been shown to have mutagenic properties.

The material basis of malignancy is as yet unknown, but there is considerable support for the view that the transition of a cell from the normal to the malignant state results from a modification in some determinative cell component which is transmitted to the cell progeny at division. There are reasons why this component should not be identified with any part of the genic system of the chromosomes, and a cytoplasmic location as a "plasmagene" has been suggested by Darlington (1948) and others. Whether nuclear or cytoplasmic, such an autoreproductive factor is likely to be protein or nucleoprotein and as such susceptible to attack and modification by agents of the "radiomimetic" class. The observation of carcinogenic activity in such agents is consistent with this conception. As with mutagenic activity the requirements for carcinogenicity are not likely to be identical with those for the cytocidal activity which leads to growth inhibition and again a modification is concerned in which cell viability must needs be preserved. In this connexion it is perhaps noteworthy that stearoyl-ethyleneimine, 248, here shown to be carcinogenic, was among those compounds with which only a small though definite increase could be produced in the percentage of anaphases in the Walker tumour showing specific chromosome changes.

*General structural considerations*

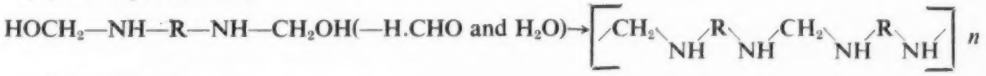
Derivatives of ethyleneimine came to be examined in this series of researches because they were known to bring about physical modifications of cellulosic and

protein fibres having much in common with those produced by certain methylolamides and epoxides. In our earlier communications, compounds of the last-named types were shown to induce characteristic mitotic disturbances in somatic cells, and it is now clear that qualitatively similar abnormalities are produced by the ethyleneimines. The association of these two properties in compounds of a single type might be coincidental, but its occurrence in all three chemical classes and its dependence in each case upon the same general structural requirements is probably significant and merits further consideration.

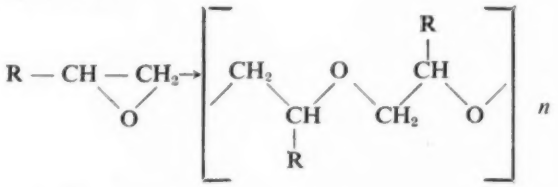
Unfortunately, precise scientific knowledge of the way in which these substances bring about their effect upon textile fibres is scanty at the present time, but it is widely accepted amongst competent technologists that one, or possibly both, of their two main reactive tendencies come into play. The first of these, the tendency to combine with nucleophilic centres, has already been mentioned (see schemes (*a*), (*b*), and (*c*) of the introduction). Except in a few cases which require separate consideration, at least two groups with reactivity of this kind must be present in each molecule of the agent if maximal effects either upon inanimate textile fibres or upon dividing cells are to be produced. From this the conclusion has been drawn that these agents produce their effect upon both systems by cross-linkage, the linkage being between polycellobiose or polypeptide units in textile fibres and, in the cell, between the protein or nucleoprotein of the chromosomes or chromatids, after the manner first proposed by Haddow and his group (Goldacre, Loveless, and Ross, 1949 ; Loveless and Revell, 1949) for the nitrogen mustards and epoxides.

The second characteristic of compounds of these three classes, to which also reference has already been made (Parts I and II) in connexion with the methylolamides and epoxides, is the facility with which they polymerize *in vitro* to give (potentially) linear structures of the following types:

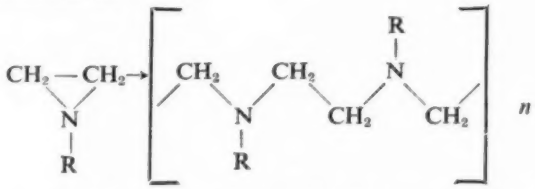
(*d*) *Methylolamides*




(*e*) *Epoxides*




(*f*) *Ethyleneimines*




Only one reactive group per molecule, or two with the methylolamides which eliminate formaldehyde, is required for polymer formation, and any other such

groups as may be present in the molecule will be accommodated initially in these polymeric units in the side chains R. Although the latter may become involved in further polymerization effects, it is known (Part II) that, in some bis-epoxides at least, the two reactive groups can behave differentially. With these compounds one epoxide group goes to form the basic repeating unit of the polymer "backbone" while the second remains in the side chain where it can then exhibit reactivity of the type shown in scheme (*b*) (page 358) above. It seems reasonable to postulate that compounds of all three types yield similar intermediate forms, with pendent groupings spaced at regular intervals along the polymer chains and capable of reacting with the nucleophilic centres of fibres or of cellular proteins or nucleoproteins. The resultant attachment of such polymer units, by numerous regularly spaced covalent bonds, either along individual polycellobiose or polypeptide chains (adlineation) or between collateral chains (cross-linkage) would be expected to bring about a much greater change in the physical and chemical properties of the substrate than would combination with unassociated molecules of the agent, as envisaged in the simpler cross-linkage hypothesis. It is suggested that a process of this nature underlies the specific biological activity common to the methylolamides, epoxides, and ethyleneimines.

Direct evidence of the occurrence of cross-linkage with or without polymer formation between cellular components or of the adlineation of polymer units thereto is inaccessible at the present time, but an appraisal of these main postulates may be made from indirect evidence obtained in the biological examination of a large number of carefully selected agents.

In so far as concerns the possibility of the formation of a polymer–cell-component complex, considerations urged in Part II in respect of the epoxides are equally applicable to the ethyleneimines. It is not inconceivable that the latter diffuse in monomeric form into cells and that there one imine radical becomes involved in the formation of a polyethyleneimine chain, while the second (and, if present, a third such radical) combines, for example, with protein or nucleoprotein. Alternatively, chemical union of the monomer with the cell-component may first occur, followed later by polymer formation through the intermolecular condensation of the remaining pendent imine groups.

In Tables I to III the activity of a number of ethyleneimine derivatives is shown in terms of both specific chromosome damage and of growth inhibition, produced in the Walker tumour. From these it has been seen that by far the most effective compounds are those containing two or three ethyleneimine radicals, and in particular derivatives of this type from pyrimidine and from 1 : 3 : 5-triazine.

In all the latter compounds, the ethyleneimino radicals are attached directly to a six-membered ring system, so that the ultimate polymer–cell-component complex, if formed, would be of the type shown in Fig. 1, with the possibility that further combination might occur between cell-components and the ethyleneimine groups attached to the ring systems shown in the upper part of the diagram. While the presence of pyrimidine or triazine nuclei would give rigidity to such a system there is no reason why ring systems *per se* should be essential, and in fact, as we have seen, certain other conjunctive groups give rise to compounds of high activity.

In Part II, it was pointed out that, within limits, the nature or dimensions of the conjunctive group linking the two essential epoxide residues had little influence

on either cytotoxic or tumour-inhibitory activity. It was suggested that this observation provided evidence against the view that these agents formed simple unimolecular cross-links and favoured the polymer hypothesis, in which the distance between the reacting groups was a function of the polyethylenoxy backbone and hence largely independent of the distance separating epoxide groups in individual molecules.

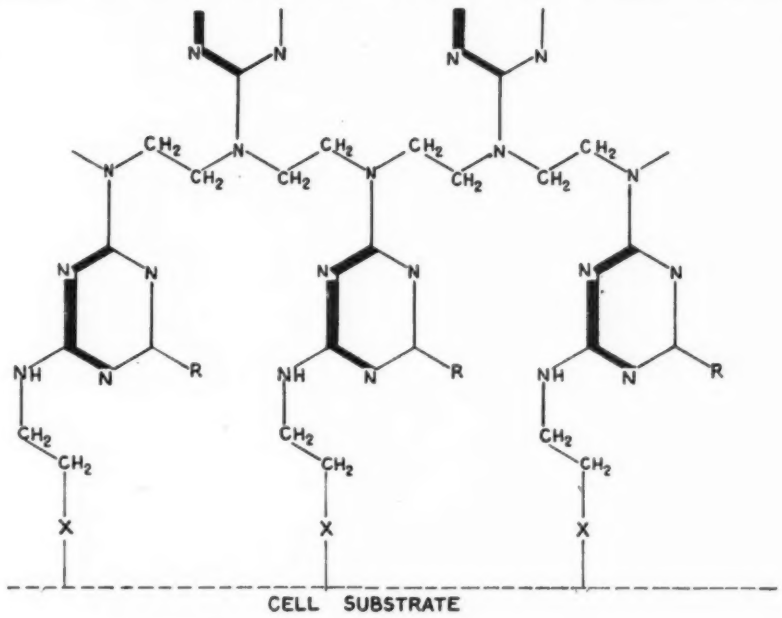



FIG. 1

Similar arguments apply in the ethyleneimine series, which provides examples of even greater permissible latitude in respect of the conjunctive group. Here active compounds range from the simple urea derivative, 275, to the bis-urea, 276, based on 4 :4′-diaminodiphenylmethane, and the octamethylene bis-sulphonamide, 291.

*Special structural considerations*

In Table I, several examples occur of mono-ethyleneimine derivatives showing appreciable tumour-inhibitory (and cytotoxic) activity. Similar observations have already been reported by workers at the Sloan-Kettering Institute (Philips, 1950), more especially with reference to 2-ethyleneimino-4 : 6-dimethoxytriazine, 272. In all compounds of this type activity is low and in many contamination with related bis-ethyleneimine derivatives in amounts sufficient to account for the observed biological effects cannot be excluded. But, as has been pointed out, the finding that 2 : 4-dinitrophenylethyleneimine, an essentially pure compound, also produces these effects compels acceptance of the occurrence of activity in monofunctional derivatives.

Biesele *et al.* (1950), in drawing attention to the activity of 2-ethyleneimino-4 : 6-dimethoxytriazine, mention the production of cytotoxic effects *in vitro* with ethyleneimine and glycidol. In addition, when administered intraperitoneally to rats, ethyleneimine was found to give rise to pancytopenia in a manner resembling the well-known effects of mustards. The failure in our laboratories and elsewhere to obtain

comparable growth-inhibiting effects in animal tumours *in situ* may perhaps be regarded as due to unfavourable properties on the part of these simple and labile molecules. Significantly these substances are well known to modify the chemical and physical properties of textile fibres. When they are used for this purpose the conditions of application are such that a large measure of polymerization must occur and the polymer chains can be visualized as lying alongside the fibre micelles, attached by end groups and attracted by residual valencies to them. A similar arrangement can be envisaged within living cells, but because of the feebler nature of the linking forces a greater concentration of agent would be needed for comparable interference with vital processes than that demanded of those, already described, which form polyreactive polymers. The residual valencies would be most effective in agents bearing the more highly polar groups in the side chain, and in this connexion attention is drawn to the notable tendency of polynitroaromatic systems to associate with various unsaturated structures to form relatively stable molecular complexes, which are now considered to be essentially ionic in character (Weiss, 1942). The effective cytotoxic unit from dinitrophenyl-ethyleneimine may well be of the form shown in Fig. 2, and linkage to cell components such as protein or nucleoprotein

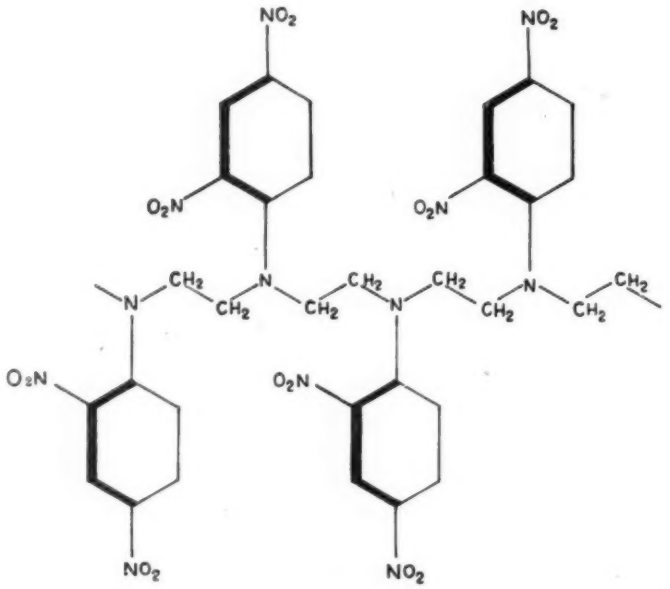


FIG. 2

may conceivably be due to association of the pendent dinitrophenyl residues with, for example, phenyl, pyrimidine, or purine nuclei in these cellular constituents. Similar considerations would apply also to the active monoethyleneimine derivatives of pyrimidine and 1 : 3 : 5-triazine mentioned above.

*Miscellaneous aspects*

The Tables of biological results show many other observations which have a bearing on the foregoing speculations, but which have not yet been discussed.

Confirmation with respect to the Walker tumour in rats of the slight but definite tumour-inhibition (Crocker mouse sarcoma 180) claimed for hexamethylmelamine (344) (Buckley, Stock, Crossley, and Rhoads, 1950) has already been mentioned. Following this, several dimethylamino analogues of other active ethyleneimino derivatives were made in order to determine the extent of this phenomenon (335, 346, 348, and 349), but only 344 and possibly 345 displayed any activity. On a purely chemical basis, two explanations appear possible. The first is that one or more of the dimethylamino groups become dehydrogenated *in vivo* to ethyleneimino, and the second that oxidation occurs leading to the intermediate formation of methylolamino or methylmethylolamino side-chains. The latter seems the more probable, particularly since it is known that *sym*-trimethyltrimethylolmelamine is a potent tumour-inhibitor (Part I, Hendry, Rose, and Walpole, 1951), and the work of the Madison group on 4-dimethylaminoazobenzene (*vide infra*) supports the metabolic course of events postulated for the dimethylamino residues.

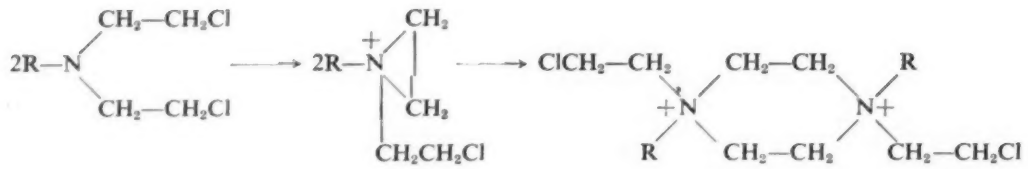
Arising from the activity shown by 2 : 4-dinitrophenylethyleneimine, a series of compounds was made carrying the same aryl residue attached to a variety of other groups (335–343). Several examples were included of bis-dinitrophenyl compounds, to see whether in these molecules the proposed ability of the polar groups to provide some form of bonding with protein or nucleoprotein would lead to a cross-linkage effect. No tumour-inhibition was observed. Similarly, the association of the dinitrophenyl radical with reactive groups such as $\beta$-bromo- and -chloro-ethylamine did not lead to active compounds. These results could all be accounted for by the absence of polymerizing properties.

Finally, attention is drawn to the urethane ethyleneimine analogue (253) which was inactive.

*Further theoretical and practical implications*

It is now desirable to see how far other substances known to have biological properties of the types under discussion can be fitted into the framework of the polymerization hypothesis, and how far such speculations are supported by experimental observation. In this connexion it is proposed to discuss only the "nitrogen mustards," the aromatic polycyclic hydrocarbons, and derivatives of azobenzene and stilbene.

*The nitrogen mustards.*—The cytotoxic, mutagenic, and carcinogenic properties of these compounds, together with their basic chemical reactions, are well established (*vide* Philips, 1950). As with the sulphur analogues, biological activity is considered to be dependent upon alkylation, mediated by the transformation of these substances into reactive cyclic ethylene-onium ions. This realization enables these compounds, and the nitrogen mustards in particular, to be discussed as special cases of the ethyleneimine type, and the close parallelism in the biological effects elicited by agents of these series lends support to this conception. The cross-linking potentialities of the nitrogen mustards as bifunctional alkylating agents in relation to these phenomena has already received consideration (Goldacre, Loveless, and Ross, 1949 ; Loveless and Revell, 1949), but the possibility of polymer formation as an intermediary stage has not so far been mentioned. Chemical studies have revealed the incidence of a certain degree of dimerization leading to (V)



(V)

(Hanby and Rydon, 1947), presumably according to the reaction sequence indicated, but polymerization to give, for example, the structure shown in Fig. 3 was not apparent, and even if it had occurred under the particular experimental conditions employed, it might not have been readily detectable. Failure to observe a polymer as a major transformation product *in vitro* does not preclude its forma-

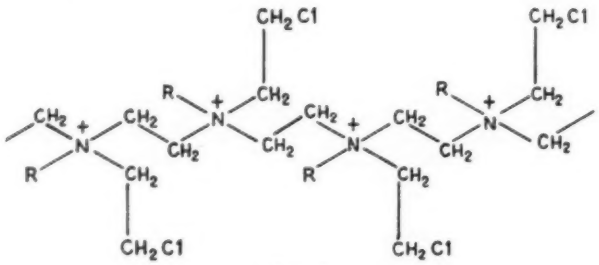


FIG. 3

tion *in vivo*, however, and the alternative possibility already proposed for the methylolamides, the epoxides, and ethyleneimines might still be applicable, namely, that the production of the drug-cell-component complex involves first the attack of single molecules of the agent upon the cell substrate, followed by intermolecular union between pendent 2-chloroethylamine residues to give the polymeric ethyleneammonium chain (Fig. 4). This view of the nitrogen mustards has the advantage

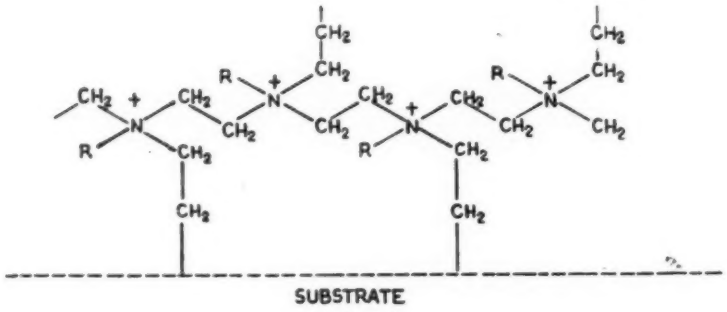



FIG. 4

that, as with the epoxides and ethyleneimines, it accommodates those members of the series in which the $\beta$-chloroethyl groups are bonded to different N atoms, even when the latter are some distance apart in the same molecule (Burchenal and Riley, 1949).

*The aromatic polycyclic hydrocarbons.*—This class of compound has been subjected to intensive chemical and biological investigation over a comparatively

long period of time, so that it is now possible to predict in empirical fashion the approximate structural requirements for carcinogenic activity. So far, the many attempts to analyse these requirements in terms of fine molecular structure have not been conspicuously successful (see Badger, 1948), but it was thought that the present hypothesis might be relevant, and indeed it has been possible to support this view by more or less direct experiment. Two characteristics of the carcinogenic cyclic hydrocarbons were selected as significant. The first was the need for an essentially planar structure of certain broadly optimum molecular dimensions. The second was the presence of a degree of chemical reactivity sufficient to provide facile conjugation with protein, for example, either directly or via the initial formation of a conjugate such as a mercapturic acid. The synthesis was then visualized, within the cell, of a protein or nucleoprotein unit carrying the hydrocarbon moieties as side-chains. The latter, by reason of their lipophilic nature, might then tend to associate into micelles, with the component flat molecules held together, perhaps in lamellar-like form, and possibly additionally interleaved by other hydrocarbon residues conjugated to a second peptide chain. The ultimate effect is shown schematically in Fig. 5. This arrangement clearly resembles that provided by the

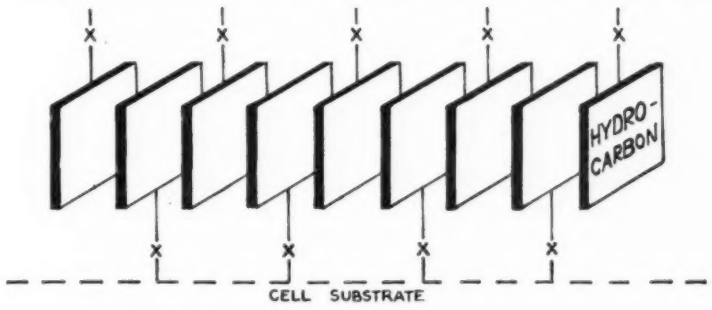



FIG. 5

reactive polymers derived from the methylolamides, epoxides, and ethyleneimines, the essential difference being the substitution of the covalent linkages between the repeating groups of the latter by residual valencies. In essence then, the carcinogenic character of the polycyclic hydrocarbons is regarded as a function of requisite chemical reactivity together with the capacity of the molecules to pack into micellar units, and it might well be that some of the differences in biological effects produced by substitution, etc., could be explained by the influence of these changes on one or both of these factors. At the moment, however, little information is available regarding the relevant physical properties of the polycyclic hydrocarbons, but acyclic hydrocarbons and their derivatives have been more intensively studied. For example, it is known that the presence of straight chain hydrocarbon groups in polar molecules (fatty acids, quaternary ammonium salts, sulphuric esters, etc.) confers micelle-forming properties. Aliphatic hydrocarbons, unlike the aromatic hydrocarbons, lack in themselves the necessary degree of chemical reactivity to combine with polypeptides and their $\alpha$-amino-acid components, a lack which on the basis of the present hypotheses would make them incapable of inducing malignancy in cells. The introduction of a single group having the necessary reactivity, however, should lead to biologically active structures. This prediction has been

tested by preparing stearoyl-ethyleneimine (248), and, as recorded above, the compound was found to exhibit marked carcinogenic properties. Clearly this result is of some theoretical importance, and current researches are directed in the first place towards a study of biological effect in relation to the known influence of chain length on the micelle-forming characteristics of polymethylene systems.*

This observation also directs attention to the possibility that similar effects might be shown by the analogous epoxide, glycidyl stearate (Part II, Serial No. 215), now under test. At the time of its preparation we considered this to be a possible product of the pyrolysis of fat according to the equation shown, along with carbon dioxide and the higher aliphatic ketones found by Peacock (1949) in his studies of the induction of tumours in the stomach of the mouse by feeding with superheated fats:

$$RCO.O.CH_2—CH(O.COR)—CH_2O.COR \longrightarrow RCO.O.CH_2—\underset{\diagdown\,O\,\diagup}{CH—CH_2} + R.CO.R + CO_2$$

*Derivatives of aminoazobenzene and stilbene*

The carcinogenic properties of nuclear and N-methylated derivatives of 4-aminoazobenzene are well known. 4-Dimethylaminoazobenzene (Butter Yellow) in particular has been subjected to intensive study by the group of workers at the McArdle Memorial Laboratory, Madison, U.S.A., and is notable for the high yield of malignant hepatomata it produces when fed to rats (Miller, Miller, and Baumann, 1945). It has also been found to combine chemically with protein of the liver. Recently Mueller and Miller (1950), in the course of an investigation of the possible significance of enzymic demethylation in this type of compound, and as a result of the isolation of formaldehyde from the reaction products of liver homogenate experiments, have suggested that the linkage to protein is through

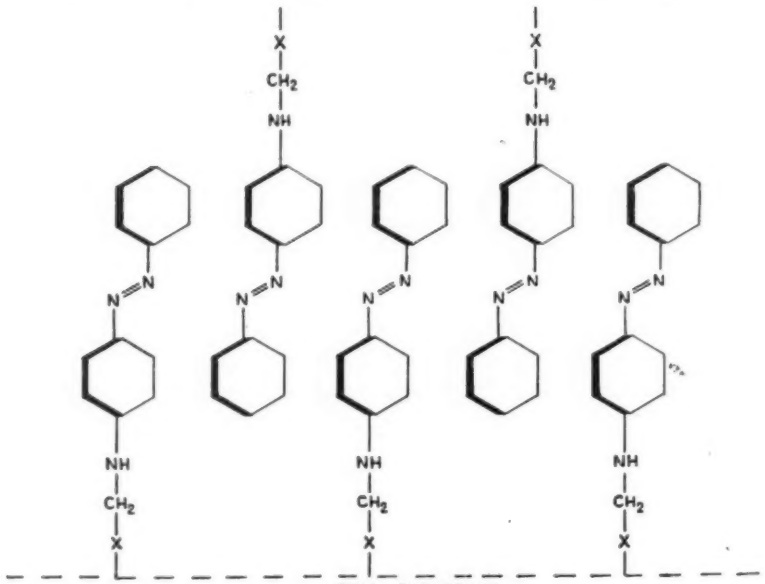



FIG. 6

*See Note (2) added in proof, p. 410.

an intermediate N-methylolaminoazobenzene. It has already been shown (Part I of this series) that an N-methylol group is capable of eliciting cytotoxic effects when suitably linked to other chemical systems, and this, together with the occurrence of the azobenzene group in a number of typical micelle-forming dyestuffs (Vickerstaff, 1950), points to a mode of action consistent with the hypothetical views now being developed. It is suggested that the attachment of the azobenzene residues to protein is followed by the orientation of the former into micelles analogous to those proposed for the polycyclic hydrocarbons (Fig. 6).

Similar arguments can be applied to the carcinogenic (and tumour-inhibitory) 4-alkylaminostilbenes of Haddow, Harris, Kon, and Roe (1948), the stilbene group again being associated in dyestuff technology with specific colloidal properties. In this connexion it is worthy of note that Koller (quoted by Boyland, 1950) has shown that 4-dimethylaminostilbene produces specific chromosome effects in the Walker tumour in rats, and we have shown that the same is true of 2′-chloro-4-dimethylaminostilbene (unpublished). So far, the analogous combination of these compounds with the protein of affected tissues has not been investigated.

## Summary

1. Preceding papers in this series have described the tumour-inhibitory and cytotoxic action of certain methylolamides and epoxides. Similar observations are here recorded in respect of over a hundred N-substituted ethyleneimine derivatives. Many of those carrying two or more ethyleneimine residues have been shown to have outstanding activity against the Walker rat carcinoma 256. The most effective compound examined has been 2 : 4 : 6-tris-ethyleneimino-*s*-triazine (322), but other triazine and pyrimidine derivatives have shown activity of a like order. Where direct comparison has been possible the pyrimidines have proved more active than their triazine analogues. Other highly active compounds include ω : ω′-bis(ethyleneimino-sulphonyl) propane (287).

2. Cytotoxic effects have been studied in Feulgen-stained preparations of tumour tissue and bone marrow from treated rats and the specific chromosome damage (fragmentation and bridge formation) produced in dividing cells evaluated quantitatively in the tumour. A correlation between the capacity to produce such changes and tumour growth inhibitory activity has been demonstrated in many instances. It is concluded, on the evidence of these and other experiments, that the tumour inhibition is a manifestation of an action directed against dividing cells in general rather than against tumour cells in particular.

3. Several pyrimidine and triazine derivatives containing only one ethyleneimine residue have been found to produce definite tumour inhibition and specific chromosome damage but only at much higher dose levels than with related bi- and tri-functional compounds. While the possibility of contamination with the latter could not be ruled out in most cases, it was unlikely in that of the active 2 : 4-dinitrophenyl-ethyleneimine (257). Ethyleneimine itself produces no such effects in the living animal.

4. A hypothesis was developed in earlier papers to explain the biological properties of the methylolamides and epoxides. The initial intermolecular condensation of these agents within the affected cells was postulated, with the formation of

polymer units with chemically reactive side chains which then combine with cell components (protein or nucleoprotein). This hypothesis is shown to be applicable to the ethyleneimines, and the relationships between structure and activity are discussed in this light. The nitrogen mustards appear as a special case of the ethyleneimine type.

5. Attempts have been made to adapt the same hypothesis to explain the carcinogenic and/or tumour-inhibitory activity of compounds of other chemical types, namely, polycyclic hydrocarbons, aminostilbenes, and aminoazobenzenes. It is suggested that conjugation of the nuclear residues of these substances with cell components (e.g., protein) occurs, followed by the association of these units into micelles simulating polymers in form. Direct evidence in favour of such a mechanism has been sought by preparing and examining stearoylethyleneimine (248) which may be regarded as another micelle-forming substance capable of reacting with cell components. This substance has been shown to be a carcinogen.

The authors are indebted to Miss D. Bonson, Miss B. Lowe, and Miss M. Durbar for technical assistance with the biological experiments.

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*Notes added in proof*

(1) The following clinical papers on 2:4:6-tris-ethyleneimino-*s*-triazine (322) have recently appeared: Karnovsky, D. A., Burchenal, J. H., Armistead, G. C., Jr., Southam, C. M., Bernstein, J. L., Craver, L. F., and Rhoads, C. P. (1951). *Arch. int. Med.*, **87**, 477. Paterson, E., and Boland, F. (1951). *Brit. J. Cancer*, **5**, (1), 28.

(2) It has now been demonstrated that, in addition to stearoyl ethyleneimine (248), myristoyl ethyleneimine ($C_{13}H_{27}CO.N(CH_2)_2$) and caproyl ethyleneimine ($C_5H_{11}CO.N(CH_2)_2$) are markedly carcinogenic.

*Brit. J. Pharmacol.* (1951), **6**, 411.

# A METHOD FOR THE ASSAY OF ANTIDIURETIC ACTIVITY

BY

MICHAEL GINSBURG

*From the Department of Pharmacology, University of Bristol*

(Received April 21, 1951)

Much recent work has been concerned with antidiuretic substances in body fluids. The method of Burn (1931) for the assay of antidiuretic activity is widely used, but has the disadvantages that it does not easily permit the estimation of the error of a single assay and that it cannot be completed in less than two to three days since the test involves a "cross-over." In the method to be described the error may be estimated from the results and the assay can be completed in four to five hours. It is based on a regimen of water administration described by Birnie, Jenkins, Eversole, and Gaunt (1949).

## METHODS

Adult male albino rats weighing approximately 200 g. were used. Rats become conditioned to water administration by stomach tube. For this reason rats were given one dose of water (5 ml./100 g. body weight) on two occasions before being used in an assay. Once conditioned the rats were not used more frequently than once in 10 days and were discarded after being used five times. Groups of 18 rats, deprived of food for 18 hours but denied free access to water for the last hour only, were placed individually in small metabolism cages; urine was collected into graduated cylinders. Each rat was given two doses of tepid water by stomach tube (each dose = 5 ml./100 g. body weight) with an interval of one hour between doses. One hour after the second administration of water the total volume of urine excreted in the preceding two hours was measured ($V_2$). At the same time a third dose of water (5 ml./100 g.) was administered and the animals injected subcutaneously with the test material. The urine output of each animal was measured thereafter at intervals of 30 minutes for 120 or 150 minutes. In order to ensure complete emptying of the bladder the animals were stimulated by prodding and handling before each administration of water and each measurement of urine volume.

The volume of urine, $V_2$, was expressed as a percentage of the amount of water administered in the first two doses. Those rats for which this value deviated from the mean for the whole group by more than 33 per cent of the mean were rejected. Since the rats were injected subcutaneously, the method of calculation adopted allowed 30 minutes for the complete absorption of the injected materials. The following formula was used:

$$\text{Percentage water excretion} = \frac{V_t - V_{30}}{3V_1 - (V_2 + V_{30})} \times \frac{100}{1}$$

where $V_1$ = the volume of water administered in each dose.
$V_2$ = the volume of urine excreted in the two hours before injection.
$V_{30}$ = the volume of urine excreted in the first 30 minutes after injection.
$V_t$ = the volume of urine excreted during the period of "$t$" minutes after injection.
($t$ = 60, 90, 120, or 150 minutes.)

That is, the percentage water excretion at a given time is the volume of urine passed from 30 minutes after injection expressed as a percentage of the water administered but not excreted 30 minutes after injection.

The antidiuretic substance used in these experiments was vasopressin (Pitressin, Parke, Davis and Co.). All solutions for injection were prepared in 0.9 per cent (w/v) sodium chloride solution. The volume injected was usually 0.2 or 0.4 ml., but larger volumes may be used provided that the volumes of the unknown and standard injections are approximately equal.

In each assay two dose levels of standard and unknown are used ; the ratio, high to low dose, being the same for standard and unknown (the value of this ratio was either 2.0 or 3.0). Before the experiment, the rats were selected for treatment by a method of random sampling. In some assays, out of the 18 rats three were selected for each standard dose of vasopressin, three for each unknown, and three for injections of saline solution. Thus there were three extra rats for the replacement of those discarded owing to exceptional diuresis during the first two hours. In most assays there was no saline-treated group, four rats being selected for each treatment with vasopressin, leaving two extra rats.

TABLE I

| | Treatment | Percentage water excretion = $Y$ | | | $\Sigma Y$ | Mean $Y$ |
|---|---|---|---|---|---|---|
| | | 60 min. | 90 min. | 120 min. | | |
| | Saline | 36.0<br>25.5<br>35.1 | 67.1<br>48.1<br>69.3 | 85.1<br>72.5<br>96.4 | | |
| Standard | $S_1$ = 0.75 milliunits Vasopressin/100 g. | 17.0<br>20.8<br>8.8 | 39.7<br>43.6<br>21.5 | 56.8<br>68.5<br>37.1 | 313.8 | 34.9 |
| | $S_2$ = 1.5 milliunits Vasopressin/100 g. | 5.0<br>14.5<br>2.8 | 14.0<br>26.6<br>8.3 | 31.7<br>47.9<br>25.0 | 175.8 | 19.4 |
| Unknown | $U_1$ | 12.4<br>9.4<br>15.4 | 31.1<br>23.2<br>31.5 | 52.8<br>44.2<br>44.6 | 264.6 | 29.2 |
| | $U_2$ | 3.4<br>4.3<br>3.2 | 6.8<br>9.3<br>13.4 | 18.1<br>23.5<br>29.6 | 111.6 | 12.4 |
| | $\Sigma Y$ | 117.0 | 269.0 | 479.8 | 865.8 | |

$$\text{Log potency ratio} = M = \frac{\Sigma Y_{\text{standard}} - \Sigma Y_{\text{unknown}}}{\Sigma Y_{\text{low doses}} - \Sigma Y_{\text{high doses}}} \times \log(\text{high dose/low dose})$$

$$= \frac{313.8 + 175.8 - 264.6 - 111.6}{313.8 + 264.6 - 175.8 - 111.6} \times 0.3010$$

$$= 0.1171$$

$$\text{Estimated potency ratio} = \frac{\text{Unknown}}{\text{Standard}} = R_E = \text{antilog } 0.1171 = 1.31$$

$$\text{Actual potency ratio} = \frac{\text{Unknown}}{\text{Standard}} = R_A \ldots\ldots\ldots\ldots = 1.20$$

## Results and Discussion

Table I shows the results obtained in a typical assay of an unknown amount of vasopressin. The percentage water excretion was taken as the response and is inversely related to the antidiuretic potency. Regardless of the time of measurement, each percentage water excretion was taken as an independent response. This is not strictly true, since the urine volumes measured at, say, 120 minutes after injection are dependent on the amounts of urine voided and measured earlier. Thus the mean response for a given animal is weighted in favour of the urine excretion in the early periods.

TABLE II

| Source of variance | Degrees of freedom | Sum of squares | Variance | Variance ratio = F | P |
|---|---|---|---|---|---|
| Deviations from parallelism | 1 | 6.3 | 6.3 | 0.1 | >0.2 |
| Regression .. .. .. | 1 | 2,352.2 | 2,352.2 | 36.8 | <0.01 |
| Standard and unknown .. | 1 | 357.2 | 357.2 | 5.6 | <0.05 |
| Times of measurement .. | 2 | 5,531.9 | 2,765.9 | 43.2 | <0.01 |
| Residual error .. .. | 30 | 1,919.2 | 63.9 | — | — |
| Total .. | 35 | 10,166.8 | — | — | — |

Standard error of $M = s_M = \sqrt{\frac{4.sy^2}{n.b^2}\left(\frac{M^2}{[\log(\text{high dose/low dose})]^2}+1\right)}$

where $sy^2$ = residual error = 63.9; $n$ = number of responses = 36

$b$ = slope of the log dose-response line $= \frac{\Sigma Y_{\text{low doses}} - \Sigma Y_{\text{high doses}}}{n/2.\log(\text{high dose/low dose})} = 53.8$

hence $s_M = \sqrt{\frac{4\times 63.9}{36\times 53.8^2}\left(\frac{0.1171^2}{0.3010^2}+1\right)} = 0.0609$

For 30 degrees of freedom $t = 2.042$ for P = 0.05

The fiducial range (P = 0.05) for $M = \pm s_M \times t = \pm 0.1243$

Since $M = 0.1171$, the fiducial limits for $M$ are 1.9928 and 0.2414, and the fiducial limits for $R_E$ are antilog $\bar{1}.9928$ and antilog 0.2414 = 0.984 and 1.74

Thus $R_E = 1.31$ with a fiducial range (P = 0.05) $\pm$ 0.38, approximately.

In the calculation of the potency ratio and error the methods described by Holton (1948) and Schild (1942) were used. The residual variance (error) was obtained through an analysis of variance (Table II) and the variance due to the different times of measurement of urine volume was eliminated. Fig. 1 shows the log dose-response lines based on the data in Table I.

Table III shows the results of 12 assays. The deviations of the estimated values of the potency of the unknown from the true values were never greater than the calculated fiducial limits (P = 0.05). In five assays (2, 4, 10, 11, and 12) the potency of the unknown was considerably different from that of the standard; in two of these assays (2 and 4) the good agreement between the true and estimated values must be regarded as fortuitous since the fiducial ranges were very wide. In practice, when an accurate assay is required and the ratio of the potencies exceeds 1.7 or is less than 0.6, a second assay should be performed in which the ratio is

TABLE III

| Assay No. | $S_1$ milliunits vasopressin per 100 g. | $S_2/S_1$ | $R_A$ = actual potency ratio unknown/ standard | $R_E$ = estimated potency ratio unknown/ standard | Approx. fiducial range (P = 0.05) | Significance of deviations from parallelism P |
|---|---|---|---|---|---|---|
| 1 | 0.75 | 3 | 1.20 | 1.20 | ±0.27 | >0.2 |
| 2 | 0.75 | 2 | 0.53 | 0.56 | 0.28 | >0.2 |
| 3 | 0.75 | 2 | 1.20 | 1.31 | 0.38 | >0.2 |
| 4 | 0.75 | 2 | 2.53 | 2.51 | 0.86 | >0.2 |
| 5 | 0.75 | 3 | 1.33 | 1.40 | 0.42 | >0.2 |
| 6 | 0.75 | 3 | 0.80 | 0.72 | 0.11 | >0.05 |
| 7 | 0.50 | 2 | 0.80 | 0.85 | 0.13 | >0.2 |
| 8 | 0.75 | 3 | 1.07 | 0.97 | 0.23 | >0.2 |
| 9 | 0.75 | 2 | 1.33 | 1.45 | 0.31 | >0.05 |
| 10 | 0.75 | 3 | 0.53 | 0.48 | 0.09 | <0.001 |
| 11 | 0.75 | 3 | 0.53 | 0.63 | 0.10 | >0.05 |
| 12 | 0.75 | 3 | 0.53 | 0.57 | 0.10 | <0.01 |

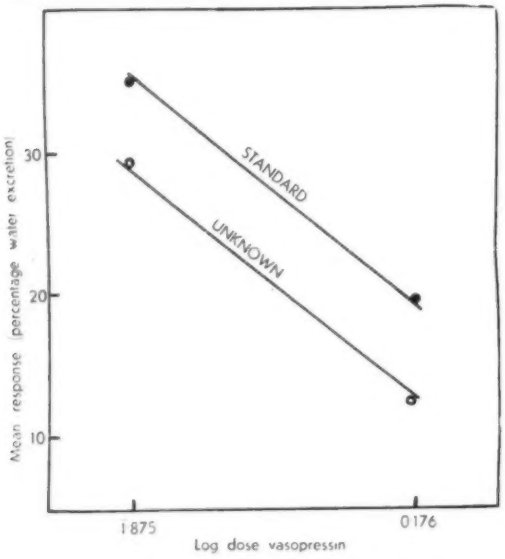


FIG. 1.—Regression lines relating response to log dose of standard and unknown. Open circles, unknown; closed circles, standard. Ordinates: Mean response (percentage water excretion). Abscissae: Log dose of vasopressin (milliunits per 100 g.).

closer to unity. The mean standard error of the method, calculated by Gaddum's (1938) method, is 8.9 per cent.

Fig. 2 shows the relationship between the log dose of vasopressin and the mean response in series of assays performed on three groups of animals. The points are fitted fairly well by straight lines, but there are significant changes in the slope and position of the lines obtained in assays performed on the same group of animals. There is a tendency for rats to become more sensitive in the later assays. Since the dose of vasopressin was related to the weight of the rat, this may be due partly to the increase in the weight of the rats and hence an increase in the absolute dose. The mean weight of the rats in the three groups at the time of the first assay was 209 g. and at the last assay 250 g. Changes in climate cannot be excluded as a possible explanation; the first assays were performed at the end of March and the last at the end of June.

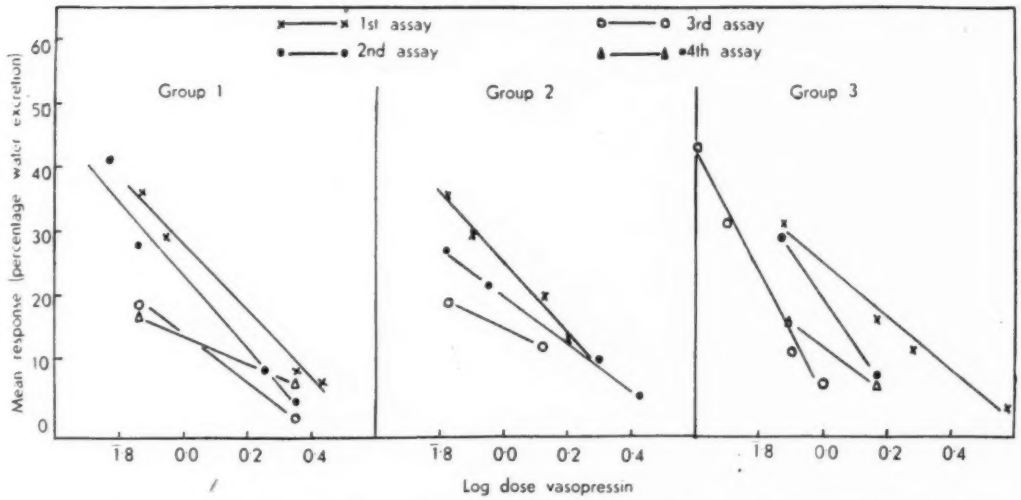


FIG. 2.—Log dose-response regression lines for 11 assays performed on three groups of rats. Ordinates: Mean response (percentage water excretion). Abscissae: Log dose of vasopressin (milliunits per 100 g.).

Sometimes the log dose-response relationship appears to show slight curvature. However, the deviations from parallelism of the standard and unknown log dose-response lines were significant in only two assays (see Table III). In these two assays the potency ratios, standard to unknown, were outside the range recommended for a precise assay; moreover, the lowest dose was 0.4 milliunits of vasopressin per 100 g. and the highest 2.25 milliunits/100 g. Over such an extended range of doses the approximation to a linear log dose-response relationship is no longer satisfactory, but log dose - log response lines are straight and parallel. According to Wood (1944) the four-point assay is still valid when the log dose-response relationship shows simple curvature: the difference between the actual and estimated potency ratios in the two assays referred to above was certainly not greater than usual.

If the variance is not independent of the magnitude of the response the analysis of variance loses its theoretical validity, but slight degrees of correlation particularly in balanced tests may be safely ignored (Emmens, 1948). In the first six assays performed the variance of the responses to each dose of vasopressin (after elimination of variance due to different times of measurement) is correlated with the magnitude of the mean response. The correlation coefficient, $r = +0.48$ ($P > 0.02$).

Though the sensitivity of the test varies, a satisfactory antidiuretic response is commonly obtained with a dose of 0.4 milliunits vasopressin per 100 g. rat; in most assays the lower dose of the standard was 0.75 milliunits, and the higher dose 1.5 or 2.25 milliunits vasopressin per 100 g. Thus the minimum total activity of the unknown required to give a satisfactory assay is about 10 milliunits; a satisfactory assay by Burn's method requires about 40 milliunits of activity. This increased sensitivity of the method is probably due to (1) a smaller number of animals being needed, (2) more complete suppression of endogenous posterior pituitary antidiuretic hormone secretion by the greater amount of water administered to the test animals,

and (3) the method of calculation in which greater weight is placed on the earlier measurements of urine volume, that is, when small doses show the greatest antidiuretic effect. The increased sensitivity of assays in which the area under the diuresis curve is taken as the response (Ham, 1943) is similarly due to greater weight being placed on the measurements of urine volume soon after injection of the antidiuretic substance.

## Summary

1. A four-point assay procedure has been applied to the assay of antidiuretic activity.

2. One assay may be completed in four to four and a half hours.

3. " Unknown " amounts of vasopressin have been assayed against vasopressin standards. The mean standard error of 12 assays was 8.9 per cent.

4. A total " unknown " activity equivalent to 10 milliunits is sufficient for a satisfactory assay.

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*Brit. J. Pharmacol.* (1951), **6**, 417.

# SOME OBSERVATIONS ON THE BIOLOGICAL PROPERTIES OF BACITRACINS A, B, AND C

BY

G. G. F. NEWTON, E. P. ABRAHAM, H. W. FLOREY, AND N. SMITH

*From the Sir William Dunn School of Pathology, Oxford*

AND

JOAN ROSS

*From the Microbiological Research Department, Experimental Station, Porton*

(Received April 23, 1951)

Newton and Abraham (1950) showed that ayfivin and bacitracin were probably identical, and that crude preparations of these antibiotics contained at least three polypeptides that were active against certain bacteria, as well as a number of other polypeptides that showed no antibacterial activity. The active peptides were called bacitracins A, B, and C. It has long been the hope that the nephrotoxicity of commercial bacitracin might be due to impurities rather than to the antibiotic itself (e.g., Meleney and Johnson, 1949). This paper records biological experiments on different constituents of crude "ayfivin."

## EXPERIMENTAL

### *Preparation of material*

A preliminary fractionation of crude ayfivin (bacitracin) was made by counter-current distribution in separating funnels, using a solvent system composed of 4 vol. amyl alcohol, 1 vol. *n*-butyl alcohol, and 5 vol. 0.05 M-potassium phosphate buffer, *p*H 7.0 (System I) (Newton and Abraham, 1950). The further purification of the individual bacitracins used for the biological studies reported in this paper was made by a series of counter-current distributions in a Craig-Post 54-chamber apparatus (Craig and Post, 1949). When bacitracins A and C were being prepared system I was used. Bacitracin B was prepared in a solvent system composed of 1 vol. amyl alcohol, 1 vol. *n*-butyl alcohol and 2 vol. 0.05 M-potassium phosphate buffer, *p*H 7.0 (System III).

The partition coefficient of A was close to unity in system I, while B had a partition coefficient of nearly one in system III.

*Bacitracin A.*—A concentrate containing 1 g. of material rich in A (85 per cent A) obtained from the preliminary fractionation was introduced equally into the first three tubes of the Craig-Post machine. A distribution of 150 transfers was then carried out in such a way that no bacitracin A was removed from the machine. At the end of the distribution the contents of the tubes that were shown to contain "pure A" (Newton and Abraham, 1950) were reconcentrated under conditions in which bacitracin A had been shown to be stable.

*Bacitracin C.*—When freshly prepared concentrates of material rich in A were redistributed in the Craig-Post apparatus about 10–15 per cent of C was obtained. However, a concentrate enriched in A which had stood in aqueous solution at *p*H 3.0 for nine months at 4° C. was found to contain little or no C. Instead, an inactive product which accounted for 10 per cent of the total material was found. The inactive product appeared in the same position on the distribution curve as bacitracin D, which is an inactive constituent of crude ayfivin having a partition coefficient of approximately 0.2 in system I.

*Bacitracin B.*—A concentrate containing about 30 per cent of bacitracin B was made from the appropriate fractions of the preliminary fractionation in system I. Two 130 transfer distributions in system III were required before the bacitracin B behaved like an essentially pure substance. In the first experiment 1.5 g. of the concentrate was loaded equally into the first four tubes of the Craig-Post machine. After 130 transfers had been completed, during which no B was withdrawn from the machine, the contents of the tubes that were shown to contain nearly pure B were reconcentrated. The concentrate, which contained about half of the B present in the original sample, was again subjected to 130 transfers in system III. This distribution yielded 160 mg. of " pure B " (10.5 per cent of the total material in the original concentrate).

*Preparation of samples for biological studies.*—It has already been shown that bacitracin is liable to lose activity when isolated in the solid state by lyophilization (Craig, Gregory, and Barry, 1949 ; Newton and Abraham, 1950).

The concentrates from the final distribution experiments were freed from potassium phosphate by extraction into *n*-butanol at *p*H 7.0, and the butanol was then removed by distillation in the presence of 0.001 N-HCl in a high vacuum. The solutions of bacitracin hydrochloride were freed from the last traces of butanol by careful extraction with *n*-hexane while still acid (*p*H 3.0) and any hexane which remained was removed by evaporation under reduced pressure. The concentration of bacitracin in the solutions was determined by the photometric ninhydrin method (Newton and Abraham, 1950). Solutions were then neutralized and adjusted to contain 0.8 per cent sodium chloride.

## Antibacterial Activity

The unit of ayfivin (bacitracin) was based on the activity of a sample of crude ayfivin hydrochloride. This material was arbitrarily stated to contain 5 units per mg. (Arriagada, Savage, Abraham, Heatley, and Sharp, 1949). A solution of ayfivin containing 1 unit per ml. produced zones of inhibition 18 to 21 mm. in diameter on plates seeded with *C. xerosis*, when assayed by the cylinder-plate method (Heatley, 1944). For the purposes of assay, solutions of bacitracin were diluted so that they contained from 0.25 to 1 unit per ml. and their activity was compared with that of the standard preparations.

The sensitivity of a number of strains of bacteria to the bacitracins was tested by the serial dilution method. *Staphylococcus* was grown in heart extract broth, and *Streptococcus* and *Corynebacterium* in 10 per cent serum broth. In each case one drop of a 24-hour culture, diluted or undiluted, was added to each of a series of tubes containing twofold serial dilutions of the bacitracins in 2 ml. of the same medium.

*Bacitracin A.*—The results obtained with a specimen of " pure " bacitracin A are shown in Table I. The material used for the titration was the same as that with which the experiments on toxicity were done, the results of which are shown in Table IV.

TABLE I

ANTIBACTERIAL EFFECT OF BACITRACIN A

| Organism | Dilution of culture used for inoculum | Highest dilution giving complete inhibition of growth for | | Lowest dilution giving no inhibition of growth for | |
|---|---|---|---|---|---|
| | | 24 hr. | 48 hr. | 24 hr. | 48 hr. |
| *Staph. aureus* (N.C.T.C. 6571) | 1 : 1,000 | 160,000 | 160,000 | 320,000 | 320,000 |
| ,, ,, (C.N. 491) .. | 1 : 1,000 | 640,000 | 320,000 | 1,280,000 | 640,000 |
| *Str. pyogenes* (C.N. 10) .. | 1 : 10 | 1,280,000 | 1,280,000 | 5,120,000 | 2,560,000 |
| *Str. haemolyticus* group C .. | 1 : 10 | 80,000 | 40,000 | 160,000 | 160,000 |
| *C. diphtheriae* (*gravis*) .. | Neat | 1,280,000 | 640,000 | 2,560,000 | 2,560,000 |

TABLE II

ANTIBACTERIAL EFFECT OF BACITRACIN B

| Organism | Dilution of culture used for inoculum | Highest dilution giving complete inhibition of growth for | | Lowest dilution giving no inhibition of growth for | |
|---|---|---|---|---|---|
| | | 24 hr. | 48 hr. | 24 hr. | 48 hr. |
| *Staph. aureus* (N.C.T.C. 6571) | 1 : 1,000 | 40,000 | 20,000 | 80,000 | 40,000 |
| ,, ,, (C.N. 491) .. | 1 : 1,000 | 320,000 | 160,000 | 640,000 | 320,000 |
| ,, ,, (penicillin-resistant) .. | 1 : 1,000 | 20,000 | 10,000 | 40,000 | 20,000 |
| *Str. pyogenes* (C.N. 10) .. | 1 : 10 | 1,280,000 | 640,000 | 2,560,000 | 1,280,000 |
| *Str. haemolyticus* group C .. | 1 : 10 | 20,000 | 20,000 | 40,000 | 40,000 |
| *C. diphtheriae* (*gravis*) .. | Neat | 1,280,000 | 1,280,000 | 2,560,000 | 2,560,000 |

*Bacitracin B.*—The results obtained with a specimen of "pure" bacitracin B are shown in Table II. The material used for the estimation was the same as that with which the experiments on toxicity were done, the results of which are shown in Table VI.

*Conclusions.*—From these results it appears that bacitracin A is about four times as potent as bacitracin B against one strain of *Staphylococcus aureus*, twice as potent against another strain, but equally powerful against a strain of *Str. pyogenes* and *C. diphtheria* (gravis).

Assayed by the cylinder-plate method against *C. xerosis* as test organism, bacitracin A had about 40 units per mg., while bacitracin B had 15 units per mg. Thus by some, but not all, criteria bacitracin A is more potent than bacitracin B.

*Mode of action* in vitro

Tubes containing serial dilutions of the bacitracins in heart broth were inoculated with one drop of an *undiluted* culture of *Staph. aureus* (N.C.T.C. 6571). The tubes were incubated at 37° C. and from time to time the number of viable organisms in each culture was estimated.

*Bacitracin A.*—Fig. 1 shows that a concentration of 1 : 40,000 or greater of bacitracin A rapidly killed all the organisms in the inoculum used, but that in a concentration of 1 : 80,000 a small proportion of the cells remained viable and eventually grew out.

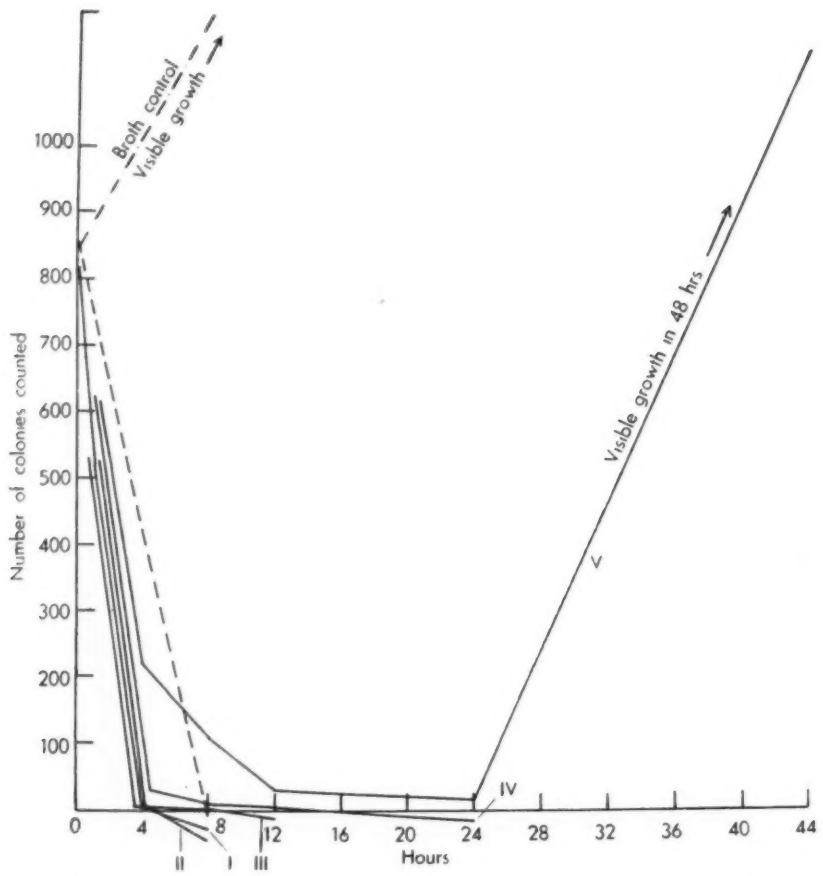


FIG. 1.—Mode of action of bacitracin A. – · – · – broth control; – – – saline control. Dilutions: I–1 : 5,000; II—1 : 10,000; III—1 : 20,000; IV—1 : 40,000; V—1 : 80,000.

*Bacitracin B.*—Fig. 2 shows that it was only with a concentration of 1 : 5,000 of bacitracin B that the culture was sterilized.

*Conclusion.*—The comparison between bacitracins A and B shows that bacitracin A is considerably more potent than bacitracin B in sterilizing cultures of *Staph. aureus.*

## TOXICITY TO MICE

Only small amounts of substance have been available for testing toxicity owing to the labour involved in separating the polypeptides with the apparatus available. It has consequently not been possible to give statistically significant figures for the LD50 of the two bacitracins, but sufficient evidence has been collected to show that the toxicity of the various fractions differs.

### BACITRACIN A

*Acute toxicity.*—All injections were given intravenously. The first experiment was done with a sample which possibly contained 15 to 20 per cent of bacitracin C

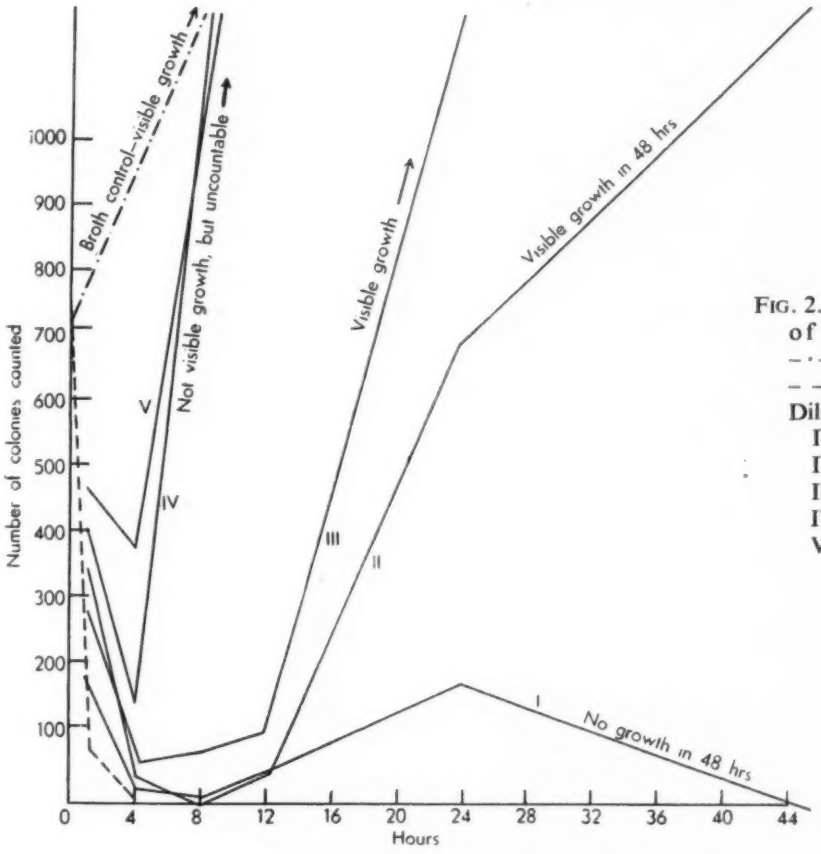


FIG. 2.—Mode of action of bacitracin B.
– · – · – broth control;
– – – saline control.
Dilutions:
I—1 : 5,000;
II—1: 10,000;
III—1: 20,000;
IV—1: 40,000;
V—1: 80,000.

but no bacitracin B. The results (Table III) showed that four out of the five mice used died after an interval of many hours.

A preparation of bacitracin A that was considered to be free from bacitracins B and C was also tested. The results are set out in Table IV. From these it seems

TABLE III

ACUTE TOXICITY TO MICE OF A PREPARATION OF BACITRACIN A CONTAINING 15 TO 20 PER CENT OF BACITRACIN C

The substance was dissolved in 0.8 per cent NaCl. All mice weighed 19 g. and were injected intravenously. The units are those defined by Arriagada *et al.* (1949), not Meleney and Johnson's units

| Dose | | | Result | Renal damage |
|---|---|---|---|---|
| Units | mg. | mg./kg. | | |
| 104.4 | 2.9 | 153 | Died about 30 hours | Grade 2c |
| 138.0 | 3.84 | 202 | Killed 73 days in good condition | |
| 155.5 | 4.32 | 227 | Died about 48 hours | Grade 2c |
| 172.8 | 4.8 | 253 | Died between 36 and 48 hours | |
| 172.8 | 4.8 | 253 | ,, ,, ,, ,, ,, | |

TABLE IV

ACUTE TOXICITY TO MICE OF BACITRACIN A CONSIDERED TO BE FREE OF BACITRACINS B AND C

The material was dissolved at a concentration of 10 mg./ml. in 0.8 per cent NaCl at *p*H 7. All mice weighed 19 g. and were injected intravenously. The units are those defined by Arriagada *et al.* (1949), not Meleney and Johnson's units

| Dose | | | Result | Post-mortem appearances | Renal damage |
|---|---|---|---|---|---|
| Units | mg. | mg./kg. | | | |
| 260 | 6.5 | 342 | Died at once | | |
| 260 | 6.5 | 342 | Killed 9 days when sick | Clear fluid in thorax and peritoneal cavity kidneys swollen with white granular surface with cysts | Grade 2b Fig. 5 Fig. 6 |
| 260 | 6.5 | 342 | Died in 22 hrs. | | |
| 260 | 6.5 | 342 | Died at once | | |
| 200 | 5.0 | 263 | ,, ,, ,, | | |
| 200 | 5.0 | 263 | ,, ,, ,, | | |
| 200 | 5.0 | 263 | Killed in good condition 21 days | Kidneys finely granular surface; spleen large | Grade 3 |
| 200 | 5.0 | 263 | ,, ,, ,, | Kidneys appeared normal; spleen large | Grade 3 |
| 200 | 5.0 | 263 | ,, ,, ,, | Kidneys finely granular surface; spleen large | Grade 3 Fig. 8 |
| 200 | 5.0 | 263 | ,, ,, ,, | ,, ,, ,, | Grade 3 |
| 160 | 4.0 | 211 | ,, ,, ,, | Kidneys appeared normal; spleen large | Grade 3 Fig. 9 |
| 160 | 4.0 | 211 | ,, ,, ,, | Kidneys and spleen appeared normal | Grade 3 |
| 160 | 4.0 | 211 | ,, ,, ,, | ,, ,, ,, | Grade 3 |
| 120 | 3.0 | 158 | ,, ,, ,, | ,, ,, ,, | N.A.D. |
| 120 | 3.0 | 158 | Died in 34 minutes | | |

probable that the LD50 would lie somewhere between 5.0 and 6.5 mg. per mouse, though one mouse out of two died soon after an injection of 3 mg. As will be seen from Table V, other mice which had received a dose previously sometimes died very soon after the injection of 3.0 mg.

*Chronic toxicity.*—The effects of repeated intravenous injection of 3 mg. bacitracin A were noted. The drug was administered in nearly all cases at 24-hour intervals (Table V).

## Bacitracin B

*Acute toxicity.*—All material was injected intravenously. Some material whose composition was not exactly known but which contained no bacitracin A was used for some preliminary experiments. Results:

1 mouse given 3.9 mg. intravenously survived 57 days in good condition
2 mice ,, 5.2 mg. ,, ,, ,, ,, ,, ,, ,,
2 ,, ,, 6.5 mg. ,, ,, ,, ,, ,, ,, ,,
of 2 ,, ,, 7.8 mg.

1 died at once and 1 survived 57 days in good condition.

TABLE V

CHRONIC TOXICITY OF BACITRACIN A

Toxicity to mice on repeated injection of equal amounts of bacitracin A (same preparation as used for experiment shown in Table IV). All mice weighed 18–19 g. and were injected intravenously. The units are those defined by Arriagada *et al.* (1949), not Meleney and Johnson's units

| Doses | | | | Results | Post-mortem appearances | Renal damage |
|---|---|---|---|---|---|---|
| No. | Total units | Total mg. | mg./kg. | | | |
| 2 | 240 | 6 | 316 | Died about ½ hr. after a 3rd dose | Kidneys pale | Grade 2c (Fig. 7) |
| 4 | 480 | 12 | 631 | Killed in good condition 24 hrs. after last dose | Kidneys appeared normal | N.A.D. |
| 1 | 120 | 3 | 158 | Died immediately after a 2nd dose | ,, ,, | Grade 1 |
| 1 | 120 | 3 | 158 | ,, ,, ,, | ,, ,, | Grade 2 |
| 4 | 480 | 12 | 631 | Killed after 21 days in good order | Kidneys finely granular; spleen large | Grade 3 |
| 3 | 360 | 9 | 474 | Killed 3 days after last dose; some air injected 1 day before death | Kidneys pale | Grade 1 (Fig. 3) |
| 4 (+1 s.c.) | 600 | 15 | 790 | Killed in good order 1 day after last injection | Kidneys appeared normal | Grade 1 |

The acute toxicity of bacitracin B was thus apparently less than that of bacitracin A, as the LD50 would appear to be somewhere between 8 and 10 mg. per mouse.

A second experiment with a sample of bacitracin B which was homogeneous and contained neither bacitracin A nor C confirmed the view that bacitracin B was less toxic than bacitracin A. The results of these experiments are given in Table VI.

TABLE VI

ACUTE TOXICITY TO MICE OF BACITRACIN B CONSIDERED TO BE FREE OF BACITRACINS A AND C

All mice weighed 20 g. and were injected intravenously. The units are those defined by Arriagada *et al.* (1949), not Meleney and Johnson's units

| Dose | | | Result | Post-mortem appearances | Renal damage |
|---|---|---|---|---|---|
| Units | mg. | mg./kg. | | | |
| 88.5 | 5.9 | 295 | Killed in good condition after 21 days | Kidney appeared normal; spleen large | Grade 3 (Figs. 10 and 11) |
| 88.5 | 5.9 | 295 | ,, ,, ,, | Kidney pale; spleen large | Grade 3 |
| 115.5 | 7.7 | 385 | ,, ,, ,, | ,, ,, ,, | ,, |
| 115.5 | 7.7 | 385 | ,, ,, ,, | Kidney finely granular; spleen large | ,, |
| 115.5 | 7.7 | 385 | ,, ,, ,, | Kidney normal; spleen large | ,, |
| 150.0 | 10.0 | 500 | ,, ,, ,, | ,, ,, ,, | ,, |
| 150.0 | 10.0 | 500 | Died in 4 minutes | | |
| 150.0 | 10.0 | 500 | Died in 1 minute | | |

TABLE VII

CHRONIC TOXICITY OF BACITRACIN B

Toxicity in mice on repeated injection of equal amounts of bacitracin B (same preparation as used for experiment shown in Table VI). All mice weighed 18–20 g. and were injected intravenously. The units are those defined by Arriagada *et al.* (1949), not Meleney and Johnson's units

| Dose | | | | Result | Post-mortem appearances | Renal damage |
|---|---|---|---|---|---|---|
| No. | Total units | Total mg. | mg./kg. | | | |
| 4 | 300 | 20 | 1,100 | Killed in good condition 24 hrs. after last injection | Kidneys large and mottled | Grade 2a (Fig. 4) |
| 6 | 267 | 18 | 890 | ,, ,, ,, | Kidneys normal | Grade 1 |
| 6 | 267 | 18 | 890 | ,, ,, ,, | Kidneys finely granular; spleen large | Early Grade 3 |

*Chronic toxicity.*—The results are shown in Table VII of giving three mice repeated daily injections of bacitracin B.

*Symptoms following injection of both bacitracins A and B*

The animals that received the larger doses from which they recovered became disoriented immediately after the injections and sometimes jumped about. A stage of excitement lasting a few seconds passed into one in which the animals were completely quiescent as though at least partially paralysed. In the course of an hour or so they gradually recovered and were able to move around freely. An interesting feature was the rapid development of great engorgement of the blood vessels of the ears, and presumably also of other parts of the body that could not be so easily seen.

There was no apparent difference between the behaviour of mice made sick by bacitracin A and of those made sick by bacitracin B.

## Bacitracin C

Only a few observations were made on a specimen rich in bacitracin C. The results shown in Table VIII suggest that bacitracin C is more toxic than the other two bacitracins, but more observations are required.

## Histological Observations on Lesions in the Kidney

The kidneys of 27 mice were examined and renal damage of varying degree was found in all but two animals.

*Group A.*—This group consisted of 12 mice that were killed or died before the tenth day after the beginning of administration. The kidneys from one mouse were normal.

*Grade 1.*—The earliest change from the normal is dilatation and epithelial damage in the ascending loop of Henle and the second convoluted tubule. Casts may or may not be present. Epithelial changes may consist of mere flattening of cells, but

TABLE VIII

ACUTE TOXICITY TO MICE OF A PREPARATION OF BACITRACIN C

All mice weighed 20 g. and were injected intravenously. The units are those defined by Arriagada *et al.* (1949), not Meleney and Johnson's units

| Dose | | | Result |
|---|---|---|---|
| Units | mg. | mg./kg. | |
| 18.75 | 0.75 | 37.5 | Killed after 57 days in good order |
| 27.5 | 1.1 | 55 | ,, ,, ,, |
| 27.5 | 1.1 | 55 | ,, ,, ,, |
| 27.5 | 1.1 | 55 | ,, ,, ,, |
| 27.5 | 1.1 | 55 | ,, ,, ,, |
| 37.5 | 1.5 | 75 | Died in 3 minutes |
| 37.5 | 1.5 | 75 | ,, 2 ,, |
| 37.5 | 1.5 | 75 | Killed after 57 days in good order |
| 37.5 | 1.5 | 75 | Found dead after 7 days |
| 75.0 | 3.0 | 150 | Died in 2 minutes |

where damage is more severe and the dilatation of the second convoluted tubule greater the epithelium may be deficient in some places. Casts with cellular debris and nuclear fragments may be present in the lumen (Fig. 3). There is little change in the first convoluted tubule or in the glomerulus.

*Grade 2.*—The next grade of damage shows alteration in the first convoluted tubule as well as more severe lesions in the second convoluted tubule (Figs. 4, 5, 6, and 7).

In the second convoluted tubules there is considerable dilatation almost amounting to cyst formation in some cases (Figs. 5). The epithelial cells are flattened, may be deficient or may show necrosis, the lumen is filled with hyaline casts and some nuclear fragments are present in the casts. Desquamated cells or nuclear fragments may form conspicuous basophil casts (Figs. 5 and 6).

The first convoluted tubules may be affected in various ways. This may take the form of an increased eosinophilia of the cytoplasm, with granularity (Fig. 4). Globules of eosinophil material may appear in the cytoplasm in the form known as hyaline droplet degeneration classed as grade 2a. In other cases (grade 2b) interstitial cells have proliferated in some areas and young connective tissue cells with young fibroblasts surround some of the first convoluted tubules and glomeruli (Fig. 5) most frequently in the superficial layers of the cortex. The tubules show evidence of collapse and the lumen is almost closed. Some glomeruli show thickening of Bowman's capsule. In kidneys with this degree of damage (grade 2a and b) mitotic figures are usually demonstrable in the epithelium.

The next group (grade 2c) represents the most severe renal damage (Fig. 7). The first convoluted tubule shows epithelial degeneration and contains granular casts while frank necrosis of the epithelium of the second convoluted tubule is present. In this group there is no evidence of epithelial regeneration, no mitotic figures being found, nor is there evidence of proliferation of connective tissue.

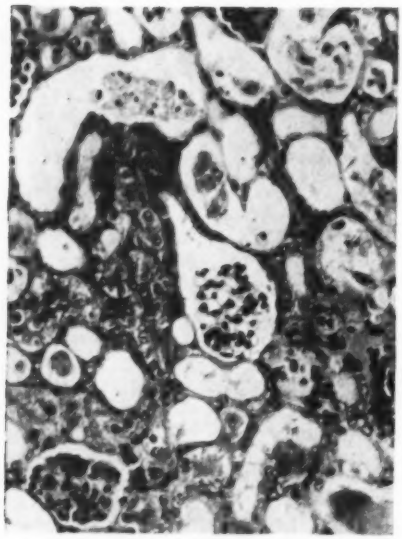

FIG. 3

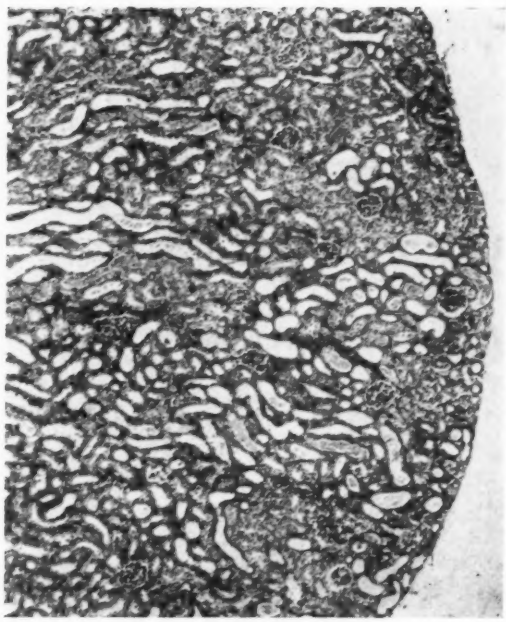

FIG. 4

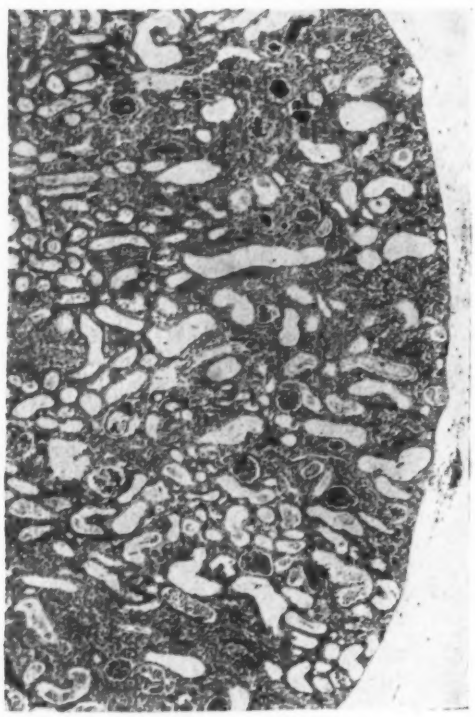

FIG. 5

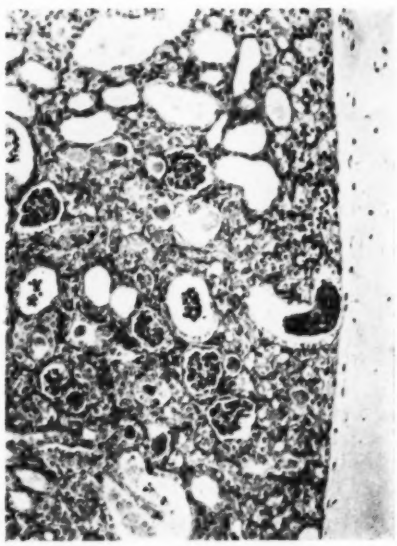

FIG. 6

FIG. 3.—See Table V. Three intravenous injections of bacitracin A at 24-hour intervals. Second convoluted tubules are widely dilated, epithelium is flattened, degenerated or absent. Casts with nuclear fragments are present. Little change is noted in the first convoluted tubules or glomeruli. Grade 1. Haematoxylin and eosin. × 305.

FIG. 4.—See Table VII. Four intravenous doses of bacitracin B at 24-hour intervals. Second convoluted and collecting tubules are dilated. Eosinophilic cytoplasm of first convoluted tubules appears dark in the photograph. Grade 2a. Haematoxylin and eosin. × 90.

FIG. 5.—See Table IV. Single intravenous dose of bacitracin A. Second convoluted and collecting tubules are widely dilated. Some casts with nuclear débris in quantity are present on the left. First convoluted tubules are compressed and there is some increase in connective tissue. Grade 2b. Haematoxylin and eosin. × 90.

FIG. 6.—Higher magnification of same kidney as in Fig. 5. Note casts in dilated second convoluted tubules and increase in connective tissue with thickening of Bowman's capsule of glomerulus on the right. Grade 2b. Haematoxylin and eosin. × 145.

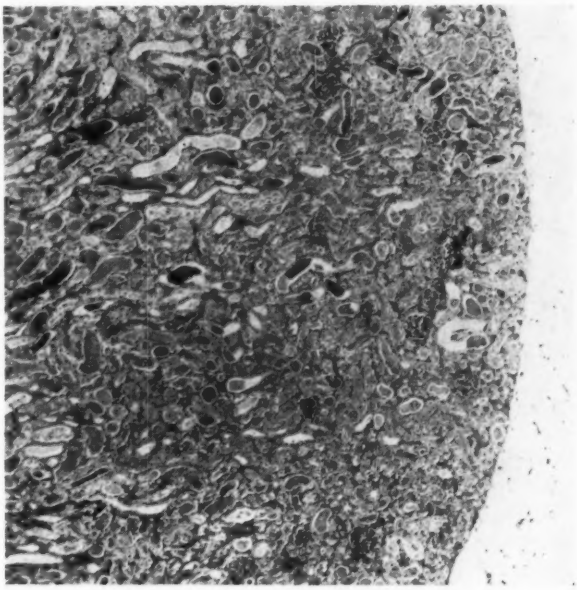

FIG. 7.—See Table V. Two intravenous doses of bacitracin A at 24-hour intervals. Widespread retention of casts in dilated second convoluted tubules. The casts are mostly hyaline but nuclear fragments are present in some. The epithelium of the second convoluted tubules is flattened or deficient. The ascending loop of Henle and collecting tubules are also affected. First convoluted tubules show epithelial degeneration. There is no proliferation of tubular epithelium or of interstitial connective tissue. Grade 2c. Haematoxylin and eosin. × 90.

The kidneys of all except one of the mice in group A can be assigned to one of these grades of damage. The exceptional animal received five doses of bacitracin B at 24-hour intervals and was killed 24 hours after the last dose. Changes in the second convoluted tubule are minimal, the cells of the first convoluted tubule show eosinophilia. In some parts of the superficial layers of the cortex there is diminution in size of the first convoluted tubules with relative increase of interstitial tissue, leading to a definite depression in the cortical surface. These patches show condensation of connective tissue round the capsule of the involved glomeruli and between the adjacent tubules, and are separated from the next patch by normal cortical tissue. The similarity of distribution and the scar-like character suggests that this animal should be classed with some of the animals killed at 21 days.

*Group B*

Fifteen mice were killed at 21 days. Nine had received bacitracin A in single doses from 3 mg. to 6.5 mg., and one mouse receiving 3 mg., showed no evidence of renal damage. The six mice that had had bacitracin B all showed renal lesions.

The lesions in the whole group follow the same pattern (grade 3). Scarred areas at present in the superficial cortex causing depression on the surface. The cortex between these areas is normal. The scarring, which involves one, two, or more nephrons, runs radially towards the medulla (Fig. 8). In some sections the damaged area includes the outer zone of the medulla. The interstitial cells in the scarred area proliferate (Figs. 9, 10, and 11). The accumulations of cells are greatest round the glomeruli. Collections of similar cells can be found in relation to the larger blood vessels in the cortico-medullary region or even more remote from the scarred area in the cortex. There is a suggestion that after bacitracin A more adult fibroblasts are present than after bacitracin B.

No exact parallel has been shown to exist between the severity of the renal damage and the amount of the antibiotic given, nor is there any constant difference

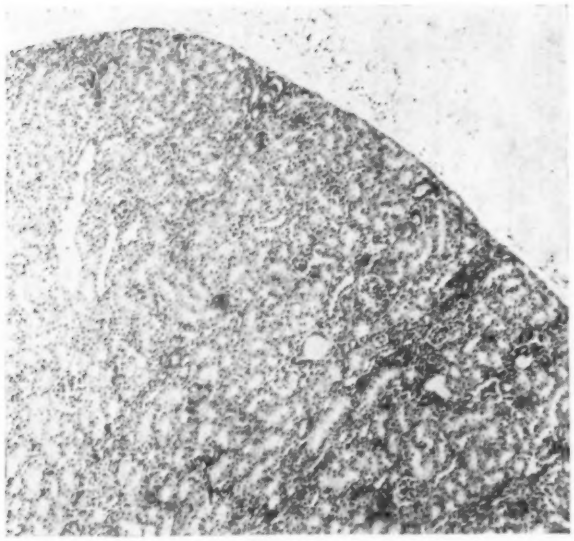

FIG 8

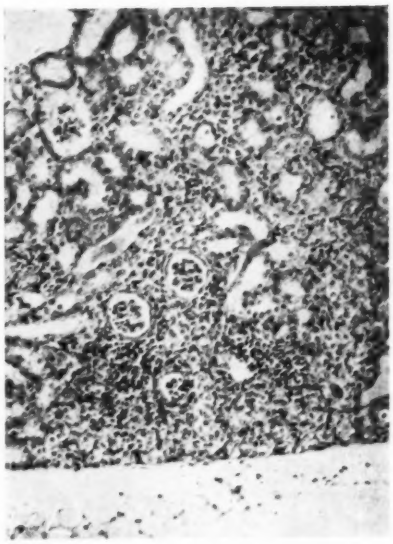

FIG. 9

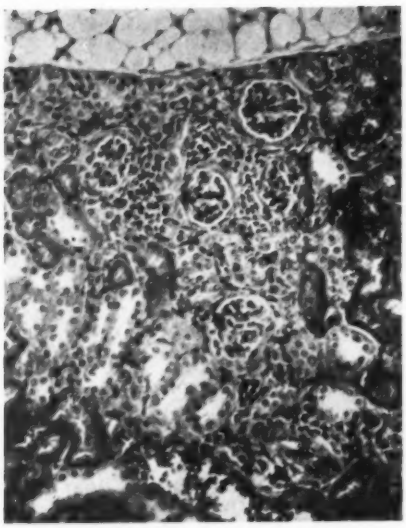

FIG. 10

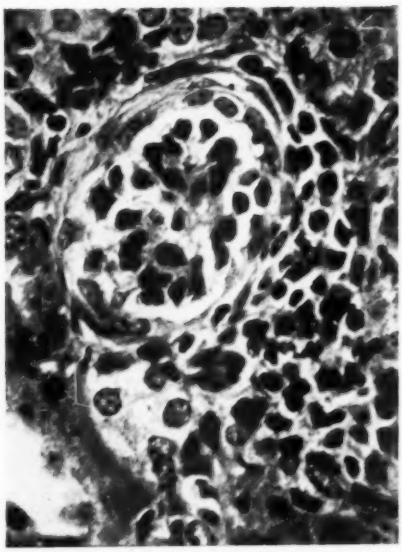

FIG. 11

FIG. 8.—See Table IV. Single intravenous dose of bacitracin A. Scarring is maximal in the outer zone of the cortex, but in this preparation the alteration can be seen to extend inwards through the cortex. (Cf. Fig. 9.) Grade 3. Haematoxylin and eosin. × 90.

FIG. 9.—See Table IV. Single intravenous dose of bacitracin A. Loss of renal tissue in the superficial layer of the cortex. Replacement by connective tissue cells with fibril formation round the glomeruli. (Cf. Fig. 8.) Grade 3. Haematoxylin and eosin. × 145.

FIG. 10.—See Table VI. Single intravenous dose of bacitracin B. There are glomerular adhesions in the subcapsular zone and an increase of connective tissue cells and fibrils. In the superficial layers of the cortex the tubules have disappeared. Grade 3. Haematoxylin and eosin. × 225.

FIG. 11.—See Table VI. Higher magnification of same kidney as in Fig. 10. A glomerulus showing an increase in the connective tissue fibrils round the capsule with adhesion of the tuft. Grade 3. Haematoxylin and eosin. × 860.

between the renal damage produced by bacitracin A and B. The initial lesion is in the second convoluted tubule, that is in the distal part of the nephron. Similar results are obtained with crude ayfivin produced by the A5 strain of *B. licheniformis* grown on the glucose ammonium-lactate medium of Hills, Belton, and Blatchley (1949). A different strain of *B. licheniformis* grown on an ammonium-lactate medium (Callow, Glover, Hart, and Hills, 1947) gives rise to an antibiotic material licheniformin II. Ayfivin and licheniformin II have been shown to be indistinguishable by counter-current distribution between solvents (Newton and Abraham, unpublished) and they produce identical lesions (Ross, unpublished).

## Discussion and Summary

The observations recorded in this paper make it reasonably certain that the nephrotoxicity of commercial preparations of bacitracin is due in part at least to the antibacterially active constituents. There is thus little hope of obtaining a preparation of bacitracin which does not have the capacity to damage the kidneys.

For therapeutic purposes it does not appear to be worth trying to separate bacitracin A from bacitracin B, for although the latter is somewhat less toxic than the former, it is less potent against certain bacterial species. The little data so far available suggest that bacitracin C has a greater acute toxicity than the other two bacitracins.

We are indebted to the Medical Research Council for grants for technical assistance and materials, to Mr. F. C. Belton, of the Microbiological Research Department, Experimental Station, Porton, for supplying the ayfivin used in these experiments, and to the Chief Scientist of the Ministry of Supply for permission to publish the results of the work done at Porton. We wish to thank Mr. E. H. Leach, of the Department of Physiology, Oxford, for photographic work, Mr. J. Kent, Mr. D. W. Jerrome, Mr. O. Boys, and Miss M. Lancaster for technical assistance, and Dr. M. A. Jennings and Miss W. M. Poynton for help in the preparation of this paper.

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# SOME BIOLOGICAL PROPERTIES OF CEPHALOSPORIN $P_1$

BY

A. C. RITCHIE,* N. SMITH, AND H. W. FLOREY

*From the Sir William Dunn School of Pathology, University of Oxford*

(Received April 23, 1951)

Cephalosporin $P_1$ is an antibiotic from a species of *Cephalosporium*. Its production is described by Crawford, Heatley, Boyd, Hale, Kelly, Miller, and Smith (1951) and its isolation and chemical properties by Burton and Abraham (1951). This substance was subjected to a fairly extensive biological investigation, because as its properties were progressively revealed it seemed possible that the substance might be of interest for therapeutic purposes. The cephalosporin used was lyophil-dried material that had been prepared by chromatography and countercurrent distribution between solvents. Its purity was appraised by analysis of the curves obtained from the distribution experiments. As will be shown later, there was little or no difference in antistaphylococcal activity between this material and crystalline cephalosporin.

## Bacteriological Investigations

### *Antibacterial activity of lyophil-dried cephalosporin $P_1$*

The sensitivity of a number of species of bacteria was tested by the serial dilution method. The staphylococci, bacilli, micrococcus, bacterium, and saprophytic mycobacteria were grown in heart extract broth ; the streptococci, neisseria, corynebacteria, and brucellae in 10 per cent serum broth ; and the erysipelothrix in Difco tryptose broth. In each case one drop of a 24-hour culture, diluted or undiluted, was added to each of a series of tubes containing twofold serial dilutions of cephalosporin in 2 ml. of the same medium. With the clostridia one drop of a four-day growth in heart extract broth was used, and with *Myco. tuberculosis* 0.2 ml. of a six-day growth in Dubos and Davis's broth was added to 1.8 ml. of medium. The presence or absence of growth was determined by naked eye observation.

It will be seen in Table I that cephalosporin $P_1$ inhibits the growth of staphylococci, corynebacteria, and *Cl. tetani* at a considerable dilution. Some organisms were inhibited at moderate dilutions, but streptococci and the Gram-negative bacteria tested were but little affected by the concentrations used.

### *Antibacterial activity of crystalline cephalosporin $P_1$*

The sensitivity of several strains of staphylococcus (N.C.T.C. 6571, C.N. 491, Hallet, Goodwin, Thurston, N.C.T.C. 8178, C.N. 1202) to crystalline cephalosporin was determined in the same way. No substantial difference was found between the activity of crystalline cephalosporin and that of the lyophil-dried material.

* Philip Walker student.

TABLE I

ANTIBACTERIAL ACTIVITY OF LYOPHIL-DRIED CEPHALOSPORIN $P_1$ *in vitro*

| Organism | Dilution of culture used for inoculum | Highest dilution giving complete inhibition of growth after incubation for: | | Lowest dilution giving no inhibition of growth after incubation for: | |
|---|---|---|---|---|---|
| | | 24 hr. | 48 hr. | 24 hr. | 48 hr. |
| *Staph. aureus* (N.C.T.C.6571) | 1 : 1,000 | 2,560,000 | 1,280,000 | 10,240,000 | 2,560,000 |
| ,, ,, (C.N. 491) | 1 : 1,000 | 2,560,000 | 1,280,000 | 10,240,000 | 2,560,000 |
| ,, ,, (Hallet) | 1 : 1,000 | 2,560,000 | 1,280,000 | 10,240,000 | 2,560,000 |
| ,, ,, (Lally) | 1 : 1,000 | 1,280,000 | 640,000 | 5,120,000 | 1,280,000 |
| ,, ,, (Welch) | 1 : 1,000 | 1,280,000 | 640,000 | 5,120,000 | 1,280,000 |
| ,, ,, (Newall) | 1 : 1,000 | 1,280,000 | 640,000 | 2,560,000 | 1,280,000 |
| ,, ,, (Broad) | 1 : 1,000 | 1,280,000 | 640,000 | 2,560,000 | 1,280,000 |
| ,, ,, (Goodwin) | 1 : 1,000 | 1,280,000 | 640,000 | 2,560,000 | 1,280,000 |
| ,, ,, (Willowchowski) | 1 : 1,000 | 640,000 | 320,000 | 1,280,000 | 640,000 |
| *Str. pneumoniae* (A 2128) | 1 : 1 | 10,000 | <10,000 | 40,000 | <10,000 |
| ,, ,, (A 2163) | 1 : 1 | 10,000 | <10,000 | 40,000 | <10,000 |
| ,, *viridans* (140) | 1 : 1 | <10,000 | <10,000 | 20,000 | <10,000 |
| ,, ,, (135) | 1 : 1 | <10,000 | <10,000 | 20,000 | <10,000 |
| ,, *haemolyticus* group A | 1 : 1 | 10,000 | <10,000 | 40,000 | <10,000 |
| ,, ,, ,, B | 1 : 1 | 10,000 | <10,000 | 40,000 | <10,000 |
| ,, ,, ,, C | 1 : 1 | 10,000 | <10,000 | 40,000 | <10,000 |
| ,, ,, ,, D | 1 : 1 | 10,000 | <10,000 | 20,000 | <10,000 |
| ,, ,, ,, G | 1 : 1 | 10,000 | <10,000 | 40,000 | <10,000 |
| ,, ,, ,, K | 1 : 1 | 10,000 | <10,000 | 40,000 | <10,000 |
| *C. diphtheriae* var. *gravis* | 1 : 1 | 2,560,000 | 1,280,000 | 5,120,000 | 2,560,000 |
| ,, ,, ,, *intermedius* | 1 : 1 | 5,120,000 | 2,560,000 | 10,240,000 | 5,120,000 |
| ,, ,, ,, *mitis* | 1 : 1 | >10,240,000 | >10,240,000 | >10,240,000 | >10,240,000 |
| ,, *xerosis* | 1 : 1 | 1,280,000 | 40,000 | 2,560,000 | 80,000 |
| ,, *hofmanni* | 1 : 1 | 5,120,000 | 1,280,000 | 10,240,000 | 2,560,000 |
| ,, *pyogenes* | 1 : 1 | 2,560,000 | 1,280,000 | 5,120,000 | 2,560,000 |
| *Cl. welchii* | 1 : 1 | | 80,000 | | 160,000 |
| ,, *tetani* | 1 : 1 | | 640,000 | | 1,280,000 |
| ,, *sporogenes* | 1 : 1 | | 20,000 | | 40,000 |
| ,, *septique* | 1 : 1 | | 80,000 | | 160,000 |
| *M. lysodeikticus* | 1 : 100 | 160,000 | 80,000 | 320,000 | 160,000 |
| *B. subtilis* | 1 : 1,000 | 80,000 | 40,000 | 160,000 | 80,000 |
| ,, *anthracis* | 1 : 1,000 | 160,000 | 80,000 | 320,000 | 160,000 |
| *N. meningitidis* | 1 : 1 | 10,000 | <10,000 | 40,000 | <10,000 |
| ,, *gonorrhoeae* | 1 : 1 | 40,000 | 40,000 | 80,000 | 80,000 |
| ,, *pharyngis sicca* | 1 : 10 | 20,000 | <10,000 | 40,000 | <10,000 |
| *Br. abortus* | 1 : 1 | <10,000 | <10,000 | <10,000 | <10,000 |
| ,, *melitensis* | 1 : 1 | <10,000 | <10,000 | <10,000 | <10,000 |
| *Bact. coli* | 1 : 1,000 | <10,000 | <10,000 | <10,000 | <10,000 |
| *Ery. monocytogenes* | 1 : 1 | 20,000 | 20,000 | 80,000 | 40,000 |
| *Myco. phlei* | 1 : 100 | | <10,000 | | <10,000 |
| ,, *smegmatis* | 1 : 100 | 80,000 | 80,000 | 160,000 | 160,000 |
| ,, *tuberculosis* (human H37)* | 1 : 1 | | 10,000 | | 20,000 |
| ,, ,, ( ,, P.N.) | 1 : 1 | | <10,000 | | <10,000 |
| ,, ,, (bovine Branch) | 1 : 1 | | <10,000 | | <10,000 |
| ,, ,, ( ,, S 182)* | 1 : 1 | | 20,000 | | 40,000 |

Where " Highest dilution . . ." and " Lowest dilution . . ." do not come next to each other in the twofold series, the intermediate tubes showed partial inhibition of growth. A blank indicates that the test was not read.

* After six days' incubation cephalosporin 1 : 10,000 did not inhibit growth.

Crystalline cephalosporin was also used to estimate the drug's action against several Gram-negative organisms not previously tested, *Bact. friedländeri, Bact. aerogenes* (63169), *Bact. aerogenes* (62691), *Salm. typhi* (B5K), *Salm. enteritidis* gaertner, *Sh. flexneri, Sh. sonnei, Sh. shigae, Ps. pyocyanea,* and *V. cholerae.* The organisms were grown in heart broth and serial dilution tests set up as before, one drop of a 24-hour culture being used as inoculum. In no case was growth affected, even by the highest concentration of cephalosporin used, 1 : 10,000. It is thus confirmed that cephalosporin is without activity against Gram-negative organisms.

*Activity of cephalosporin against penicillin-resistant staphylococci*

Table I shows that cephalosporin $P_1$ was highly active against penicillin-resistant staphylococci. The Willowchowski strain was resistant to concentrations of penicillin up to 500 units per ml., Goodwin to concentrations up to 250 units, and Lally, Welch, Newell, and Broad to concentrations up to 100 units per ml. The N.C.T.C. 6571, C.N. 491, and Hallet strains were highly sensitive to penicillin.

*Effect of inoculum size*

The effect of varying the size of the inoculum was investigated in similar serial dilution tests, a 24-hour culture being diluted with water as required. Table II shows that the size of the inoculum made a considerable difference to the end-point

TABLE II

EFFECT OF INOCULUM SIZE ON ANTIBACTERIAL ACTIVITY OF CEPHALOSPORIN $P_1$

| Organism | Dilution of culture used for inoculum | Highest dilution giving complete inhibition of growth after incubation for: | | Lowest dilution giving no inhibition of growth after incubation for: | |
|---|---|---|---|---|---|
| | | 24 hr. | 48 hr. | 24 hr. | 48 hr. |
| *Staph. aureus* (N.C.T.C. 6571) | 1 : 1 | 320,000 | 160,000 | 640,000 | 320,000 |
| | 1 : 1,000 | 2,560,000 | 1,280,000 | 10,240,000 | 2,560,000 |
| | 1 : 1,000,000 | 5,120,000 | 2,560,000 | 10,240,000 | 5,120,000 |
| *C. diphtheriae* var. *gravis* | 1 : 1 | 2,560,000 | 1,280,000 | 5,120,000 | 2,560,000 |
| | 1 : 100 | 2,560,000 | 1,280,000 | 10,240,000 | 2,560,000 |
| | 1 : 10,000 | >40,960,000 | 2,560,000 | >40,960,000 | 5,120,000 |

when *Staph. aureus* (N.C.T.C. 6571) was used, but that it was unimportant with *C. diphtheriae* var. *gravis* when the results were read after 48 hours' incubation.

*Effect of serum*

Concentrations of cephalosporin of 1 : 10,000, 1 : 50,000, and 1 : 250,000 in water, in 50 per cent serum, and in 90 per cent serum were incubated at 37° C. for five hours, and the antibacterial activity of the solutions against *Staph. aureus* (N.C.T.C. 6571) tested by the cylinder-plate method. From the zone size it appeared that in both 50 per cent and 90 per cent serum the activity was reduced by about 60 per cent.

*Development of resistance by staphylococci* in vitro

*Staph. aureus* (N.C.T.C. 6571) was cultivated in tubes of broth containing serial dilutions of cephalosporin. After 24 hours cocci from the tube with the highest

concentration that allowed visible growth were subcultured into another series of tubes containing serial dilutions. After three such subcultures the staphylococci were not inhibited by cephalosporin at a concentration of 1 : 25,000. It was thus clear that they had rapidly acquired considerable resistance to the substance *in vitro.*

In view of the work to be described later it is interesting to note that when treated in a similar way *Staph. aureus* (C.N. 491) acquired no resistance to aureomycin hydrochloride when grown in its presence, even after six subcultures. This finding agrees with that of Price, Randall, and Welch (1948).

*Mode of action* in vitro

Tubes containing serial dilutions of cephalosporin in 2.0 ml. of broth were inoculated with one drop of an undiluted 24-hour culture of *Staph. aureus* (N.C.T.C. 6571). The tubes were incubated at 37° C., and from time to time the number of viable organisms in each culture was estimated. Fig. 1 shows the results. A concentration of cephalosporin of 1 : 400,000 or less inhibited growth only temporarily, but with a concentration of 1 : 200,000 or more no viable organisms were found after 24 hours.

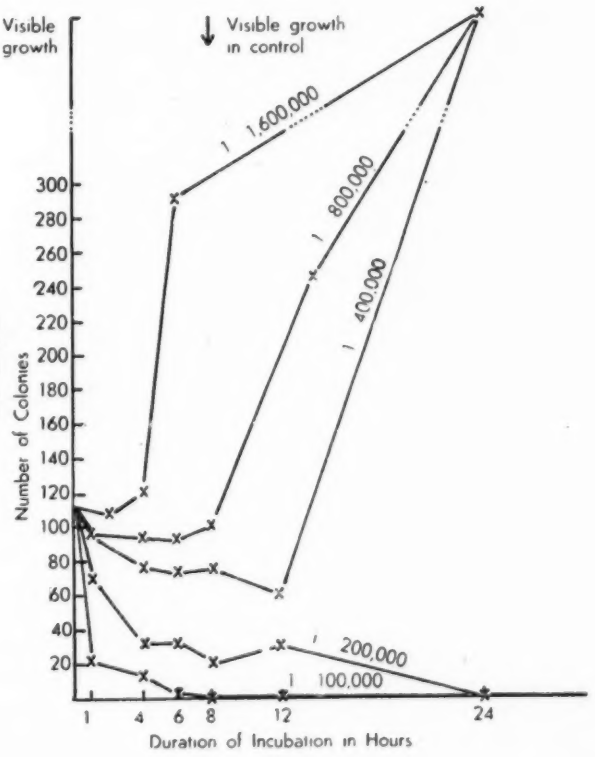


FIG. 1.—Mode of action of cephalosporin $P_1$ *in vitro*. The number of staphylococci surviving after cultivation in media containing cephalosporin. Each point is the average of three measurements.

This means that under the conditions of the test with the small inoculum used cephalosporin is bactericidal at a concentration of 1 : 200,000 or more, while in lower concentrations it is bacteriostatic.

*Comparison with helvolic acid, aureomycin, and terramycin*

There are certain similarities between the biological behaviour of helvolic acid (Florey, Chain, Heatley, Jennings, Sanders, Abraham, and Florey, 1949, page 332) and cephalosporin, and, as will be seen later, it became desirable to compare the action of cephalosporin *in vivo* with that of aureomycin and terramycin. Some comparative *in vitro* tests were therefore made.

The sensitivity of two strains of staphylococcus to helvolic acid, aureomycin hydrochloride, and terramycin hydrochloride was tested by the dilution method in the same way as was their sensitivity to cephalosporin. The highest dilution of helvolic acid completely inhibiting growth after 48 hours' incubation was 1 : 160,000 for *Staph. aureus* (C.N. 491) and 1 : 320,000 for *Staph. aureus* (N.C.T.C. 6571). Both these strains were inhibited by dilutions of terramycin up to 1 : 2,560,000. Aureomycin is destroyed when in solution in the medium used for these tests, and so in the tests with this substance readings were made earlier. The growth of both strains was inhibited for 12 hours by concentrations up to 1 : 1,280,000 and for 24 hours by concentrations up to 1 : 640,000 for *Staph. aureus* (C.N. 491) and up to 1 : 1,280,000 for *Staph. aureus* (N.C.T.C. 6571). If these findings are compared with those shown in Table I it is seen that the antistaphylococcal action of cephalosporin *in vitro* is stronger than that of helvolic acid, but of the same order as that of aureomycin and terramycin.

*C. diphtheriae* var. *gravis* was found to be completely inhibited by dilutions of helvolic acid up to 1 : 5,120,000.

A comparison with Table I in the paper by Chain, Florey, Jennings, and Williams (1943) shows that cephalosporin has a similar antibacterial range to that of helvolic acid.

## Pharmacological Investigations

*Acute toxicity*

*To mice.*—As supplies of material were limited, only the effects of intravenous injection were determined. Cephalosporin $P_1$ is not freely soluble, the maximum concentration obtainable in isotonic saline at *p*H 7 being 10 mg./ml. This necessitated the injection of relatively large volumes of fluid. The injection of 1.5 ml. of a saturated solution, i.e., 15 mg. of substance, intravenously into mice weighing 20 g. caused the death of the four mice used, two after 16 hours, one after 19 hours, and one after 22 hours. All had epileptiform convulsions immediately after the injection, from which they passed into a semi-comatose condition. The injection of 1.5 ml. of isotonic saline did not affect two control animals.

Thirteen mice of 20 g. weight were given 1 ml., i.e., 10 mg. Immediately after injection they became ill and were semi-comatose, but eleven gradually improved during the six hours after the injection and eventually recovered fully. They were well at the end of three weeks when killed for histological examination. No lesions attributable to the cephalosporin were found in liver, kidney, or spleen. Of the two that died, one lived for 16 and one for 28 hours. Thus the LD50 after intravenous injection is between 10 and 15 mg. per mouse, i.e., between 500 and 750 mg./kg.

*To leucocytes.*—The effect of cephalosporin on human leucocytes was determined by the method of Abraham, Chain, Fletcher, Florey, Gardner, Heatley, and Jennings

(1941). Concentrations of 1 : 220 to 1 : 880 killed about half the cells in four hours, but with concentrations of 1 : 1,000 or less the survival time did not differ from that of the cells in control preparations, most cells surviving more than six hours.

*To tissues.*—No gross abnormality was seen at the site of injection in mice killed from one to eleven hours after a single subcutaneous dose of 1 or 2 mg. of cephalosporin, nor any lesion of the gut in mice given a single dose of 1, 2.5, or 5 mg. by mouth in 0.6 ml. of water from one to fifteen hours before death. Mice given 5 mg. aureomycin hydrochloride by mouth in the same volume of fluid showed injection of the small intestine when killed after five and seven hours, and mice given 5 mg. terramycin hydrochloride slight " blushing " of the gut when killed after nine hours.

*Chronic toxicity*

*To mice.*—Five mice were given 4 mg. cephalosporin a day for four days by subcutaneous injection, 1 mg. at 0900 hours, 1 mg. at 1600 hours, and 2 mg. at 2300 hours. Three weeks after the first injection, when killed for histological examination, all were still well. No animal showed any gross lesion *post mortem*, nor any histological abnormality in liver, kidney, or spleen that could be attributed to the cephalosporin.

Cephalosporin, 5 mg. 12-hourly for 5½ days, was given by mouth to five mice. No abnormalities were noted, and all were well when killed three weeks after the first dose. No gross lesion was found *post mortem*, nor any histological abnormality in liver, kidney, or spleen that could be attributed to the cephalosporin.

*To tissues.*—In the animals used for the chronic toxicity tests no lesion was found at the site of injection in those that had been given cephalosporin subcutaneously, and no abnormality was detected in the gut of those given the drug by mouth.

*Effect on blood pressure*

Cephalosporin $P_1$ injected intravenously in doses of up to 200 mg. in 20 ml. affected neither the blood pressure nor the respiration of a decerebrate cat weighing 2.8 kg.

*Effect on smooth muscle*

The effect of adding cephalosporin $P_1$ to a bath in which an isolated guinea-pig uterus was contracting rhythmically was the same for all concentrations tested from 1 : 100,000 to 1 : 10,000. At first the rate and excursion of the contractions were increased, and then, after a few minutes, the contractions became slower and of great amplitude. The original type of contraction was restored after removal of the cephalosporin.

*Absorption*

*After subcutaneous injection.*—The presence of cephalosporin in the blood was detected either by the slide method of Garrod and Heatley (1944) or by the hole-plate method (Florey *et al.*, 1949, page 130). The latter method gave adequate information, and as it is easier to perform was generally used. Mice weighing 18 to 20 g. were killed at various intervals after the injection of cephalosporin, and

undiluted serum from the blood was collected and placed in holes punched in a plate sown with *Staph. aureus* (C.N. 491). The zones of inhibition were read after 18 hours' incubation at 37° C. The results are shown in Fig. 2. Cephalosporin was absorbed from the subcutaneous tissues to produce easily detectable concentrations in the blood. As a very rough approximation it can be taken that a zone of

FIG. 2.—Serum levels in mice after the injection of cephalosporin $P_1$ subcutaneously. Lower curve (△---△) after 1 mg.; upper curve (O———O) after 2 mg. Blood serum taken at different times after injection was tested for activity in hole-plates seeded with *Staph. aureus*. (N.B. A reading of 6 mm. represents the diameter of the hole and indicates no inhibition.) Each point in the 1 mg. curve is the average of measurements on the sera of two mice; the hollow circles on the 2 mg. curve are measurements from one mouse, the solid circles measurements on the pooled sera of three mice.

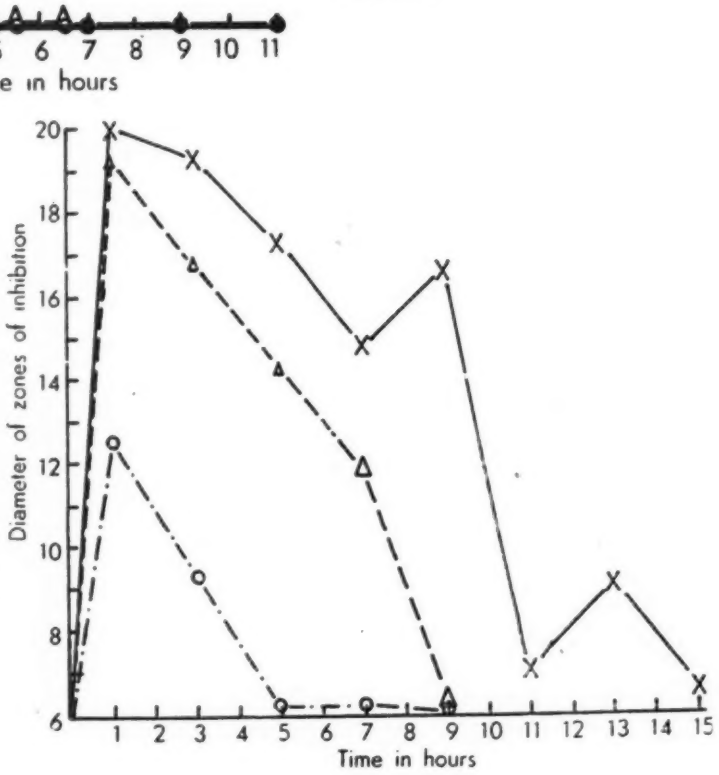


FIG. 3.—Serum levels in mice after the administration of cephalosporin $P_1$ by mouth. Lowest curve (o-.-.-o) after 1 mg.; middle curve (△- - -△) after 2.5 mg.; highest curve (X———X) after 5 mg. Activity was measured by the hole-plate method using 6 mm. holes and plates seeded with *Staph. aureus*. Each point on the curves is the average of measurements on four mice, except the last three points on the 5 mg. curve which are the average for three mice.

inhibition of diameter 18–19 mm. represents a concentration of cephalosporin of about 1 : 100,000 in the serum.

*After oral administration.*—Single doses of 1, 2.5, and 5 mg. dissolved in 0.6 ml. of water were administered by stomach tube to mice weighing 18 to 20 g. The mice were killed at intervals and their sera assayed as before. Fig. 3 shows the results.

About 15 hours after the administration of 5 mg. cephalosporin by mouth none was present in the stomach and little in the jejunum, but a considerable amount was present in the colon. There was little in any of these situations after the administration of 1 or 2.5 mg.

It is thus clear that cephalosporin is readily absorbed from the gastro-intestinal tract, leading to the appearance of well-sustained concentrations in the blood.

*Comparison of absorption of cephalosporin with aureomycin and terramycin*

As will be seen later, it became desirable to compare the effects of aureomycin, terramycin, and cephalosporin on experimental staphylococcal infection in mice.

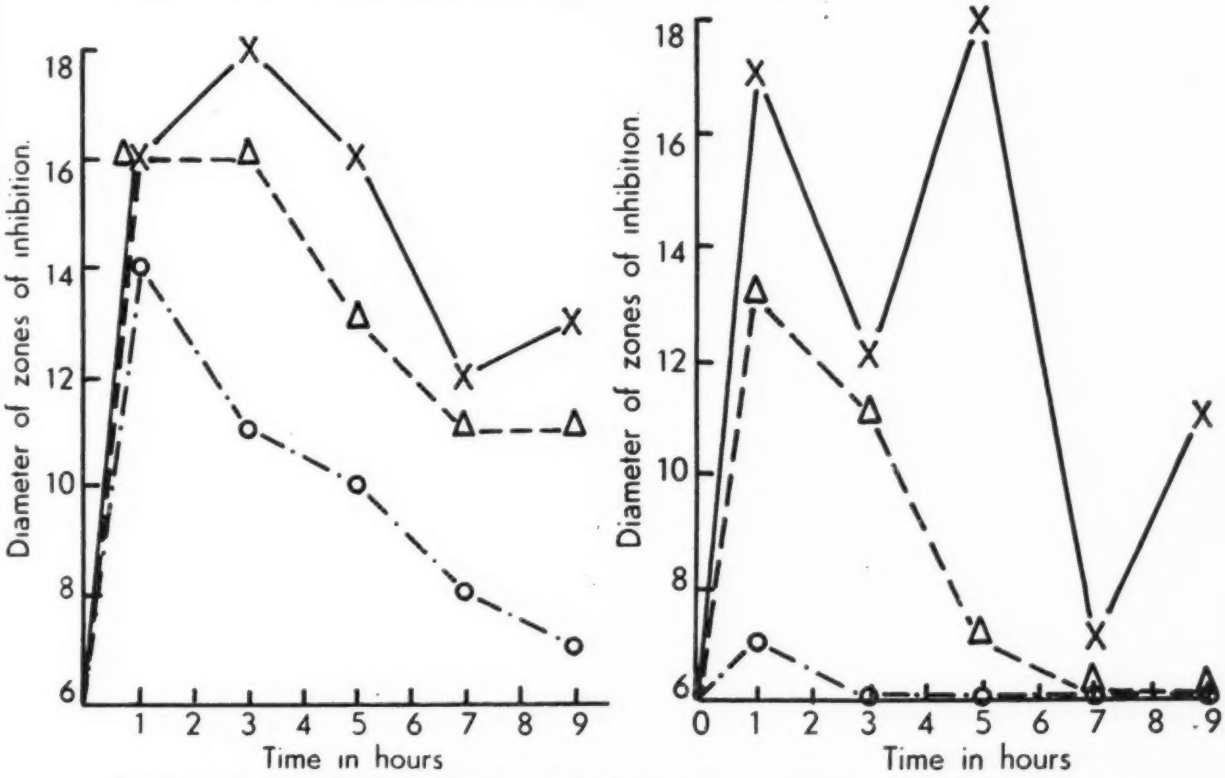


FIG. 4.—Serum levels in mice after the administration of aureomycin by mouth. Lowest curve (o–.–.–o) after 1 mg.; middle curve (△– – –△) after 2.5 mg.; highest curve (X———X) after 5 mg. Activity was measured by the hole-plate method using 6 mm. holes and plates seeded with *Staph. aureus*. Each point represents the measurement obtained from the serum of one mouse.

FIG. 5.—Serum levels in mice after the administration of terramycin by mouth. Lowest curve (o–.–.–o) after 1 mg.; middle curve (△– – –△) after 2.5 mg.; highest curve (X———X) after 5 mg. Activity was measured by the hole-plate method using 6 mm. holes and plates seeded with *Staph. aureus*. Each point represents the measurement obtained from the serum of one mouse.

Comparisons were made of absorption of the three substances when given by mouth under the same conditions. Figs. 4 and 5 show the results.

It would appear that under the conditions of the experiment cephalosporin and aureomycin are retained in the blood for about the same time, slightly longer than is terramycin.

*Excretion*

*In the urine.*—Twenty-one mice of 20 g. weight were given a single subcutaneous injection of 2 mg. cephalosporin, and the urine excreted in the next five hours collected. The amount of cephalosporin excreted in the urine was measured by estimating the urinary concentration of the drug by the hole-plate method. The average amount excreted in five hours was less than 0.01 mg., a very small proportion of the injected dose.

The urine was collected from two decerebrate cats used for investigating the effect of cephalosporin on the blood pressure. Although it is not possible to give accurate quantitative results from these experiments, they agree with those obtained in mice in that only a small amount appeared in the urine. The first cat, which received 200 mg. intravenously in divided doses during 3 hours 53 minutes, secreted less than 1 mg. of cephalosporin in the urine collected during the 4½ hours after the first injection, and the second cat, which received 350 mg. intravenously in 3 doses during 43 minutes, secreted about 2 mg. in the 3 hours after the first injection.

*In the bile and by the gastro-intestinal tract.*—Bile was also collected from the two cats just mentioned. The cat that was given 200 mg. cephalosporin during 3 hours 53 minutes secreted about 1.2 mg. of cephalosporin in the 4½ hours after the first injection, and the cat that was given 350 mg. during 43 minutes about 2.8 mg. in the 3 hours after the first injection.

Ten mice that were given a single subcutaneous injection of 2 mg. cephalosporin were killed 5 hours later, and the stomach, a length of jejunum, and a length of colon were each washed out with 0.25 ml. saline. Activity was detected by the hole-plate method in the colonic washing from every mouse, in the jejunal washing from all but one, and in the stomach washing from four. It is possible therefore that in mice cephalosporin is excreted by the alimentary canal, but it is more likely that its presence there is due to excretion in the bile.

Thus the relatively rapid elimination of cephalosporin from the blood does not seem to be adequately accounted for by urinary or biliary secretion. Further attempts to ascertain its fate in the body were not made.

## Protection Experiments

The staphylococcus is among the organisms most sensitive to the action of cephalosporin $P_1$, and though the activity of the drug is reduced in the presence of serum it is not abolished. In this cephalosporin resembles aureomycin, the efficacy of which is also reduced in the presence of serum (Chandler and Bliss, 1948). Terramycin is less affected (Bliss, Warth, and Chandler, 1950). The foregoing experiments made it clear that cephalosporin was sufficiently non-toxic to mice to administer for a long time in sufficient dosage to maintain a level in the blood that inhibits the growth of the staphylococcus *in vitro*. There were, therefore,

reasonable grounds for supposing that it might suppress the growth of the staphylococcus in the body. Experiments were done to test this supposition, using the strain of *Staph. aureus* (C.N. 491) that had been used to estimate the level of cephalosporin in the blood of normal animals.

A 24-hour culture was diluted as required and one vol. of the diluted culture added to nine vols. of a 7 per cent suspension of mucin. The mice were infected by injecting 0.5 ml. of this suspension intraperitoneally. As the virulence of the organism varied from time to time different dilutions of the culture were used in different experiments. An approximate LD50 determined on small numbers of animals is given in each Table of results.

In the first experiment the cephalosporin was made up at a strength of 5 mg. per ml. Each mouse was given three subcutaneous injections daily, 1 mg. of cephalosporin at 0900 hours, 1 mg. at 1600 hours, and 2 mg. at 2300 hours, beginning one hour after infection. Table III shows that the cephalosporin increased the length of life but not the survival rate.

TABLE III

ANTISTAPHYLOCOCCAL ACTION OF CEPHALOSPORIN $P_1$ *in vivo*

4 mg. given daily in three doses subcutaneously. LD50 was between 1 : 10,000 and 1 : 100,000 dilutions. Dilution of culture: 1 : 1,000

| | Control | Cephalosporin |
|---|---|---|
| Mice alive at start | 10 | 10 |
| ,, ,, after 24 hours | 1 | 10 |
| ,, ,, ,, 48 ,, | 1 | 9 |
| ,, ,, ,, 3 days | 1 | 9 |
| ,, ,, ,, 4 ,, | 1 | 7 |
| ,, ,, ,, 5 ,, | 1 | 3 |
| ,, ,, ,, 6 ,, | 1 | 0 |
| ,, ,, ,, 7 ,, | 1 | |
| ,, ,, ,, 14 ,, | 1 | |
| ,, ,, ,, 21 ,, | 1* | |

*Gross staphylococcal lesions in survivor.

Table IV shows the findings in the second experiment. Cephalosporin was given subcutaneously in the same way as before and in the same dose (4 mg. a day), but the size of the inoculum of staphylococcus was varied. As in the first experiment, the drug increased the length of life but not the number of animals surviving. Decreasing the inoculum size a thousandfold did not affect the result.

It seemed likely that the dose of cephalosporin given in these experiments was insufficient, for it had been shown (Fig. 2) that a single dose of 1 mg. subcutaneously produced a detectable concentration in the serum for only two hours or so, and a single dose of 2 mg. a level detectable for only about three and a half hours. In these protection experiments there was an interval of seven hours after each dose of 1 mg. and an interval of ten hours after the dose of 2 mg. A further experiment was therefore done in which 5 mg. of cephalosporin was given by mouth every 12 hours. A single oral dose of this size gave a detectable serum concentration for about 15 hours (Fig. 3), and so repeated doses should maintain a detectable level throughout. The results of the experiment are shown in Table V. Under

TABLE IV

EFFECT OF INOCULUM SIZE ON ANTISTAPHYLOCOCCAL ACTION OF CEPHALOSPORIN $P_1$ *in vivo*

4 mg. given daily in three doses subcutaneously. LD50 was at a dilution of about 1 : 1,000,000

| Dilution of culture: | 1 : 1,000 | | 1 : 5,000 | | 1 : 10,000 | | 1 : 50,000 | | 1 : 100,000 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Cephalosporin | Control | Cephalosporin | Control | Cephalosporin | Control | Cephalosporin | Control | Cephalosporin |
| Mice alive at start .. | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, after 24 hours | 2 | 5 | 2 | 5 | 4 | 5 | 3 | 5 | 2 | 5 |
| ,, ,, ,, 48 ,, | 0 | 4 | 1 | 5 | 0 | 5 | 2 | 1 | 0 | 4 |
| ,, ,, ,, 3 days | | 2 | 1 | 5 | | 5 | 2 | 1 | | 4 |
| ,, ,, ,, 4 ,, | | 1 | 0 | 5 | | 4 | 1 | 1 | | 4 |
| ,, ,, ,, 5 ,, | | 0 | | 4 | | 1 | 0 | 1 | | 1 |
| ,, ,, ,, 6 ,, | | | | 3 | | 1 | | 1 | | 1 |
| ,, ,, ,, 7 ,, | | | | 3 | | 0 | | 1 | | 1 |
| ,, ,, ,, 14 ,, | | | | 2 | | | | 1 | | 1 |
| ,, ,, ,, 21 ,, | | | | 2* | | | | 1* | | 1* |

* No staphylococcal lesions in survivors.

TABLE V

ANTISTAPHYLOCOCCAL ACTION OF CEPHALOSPORIN $P_1$ *in vivo*

5 mg. given by mouth every 12 hours for 5½ days. LD50 was at a dilution between 1 : 100,000,000 and 1 : 1,000,000,000

| Dilution of culture: | | 1 : 10,000 | | 1 : 100,000 | | 1 : 1,000,000 | | Combined result | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | Cephalosporin | Control | Cephalosporin | Control | Cephalosporin | Control | Cephalosporin |
| Mice alive at start .. | | 10 | 10 | 10 | 10 | 10 | 10 | 30 | 30 |
| ,, ,, after 24 hours | | 3 | 8 | 2 | 8 | 2 | 10 | 7 | 26 |
| ,, ,, ,, 48 ,, .. | | 2 | 7 | 2 | 7 | 2 | 10 | 6 | 24 |
| ,, ,, ,, 3 days .. | | 2 | 7 | 2 | 6 | 2 | 10 | 6 | 23 |
| ,, ,, ,, 4 ,, .. | | 2 | 6 | 2 | 6 | 2 | 9 | 6 | 21 |
| ,, ,, ,, 5 ,, .. | | 2 | 6 | 2 | 6 | 2 | 9 | 6 | 21 |
| ,, ,, ,, 6 ,, .. | | 2 | 6 | 2 | 6 | 2 | 9 | 6 | 21 |
| ,, ,, ,, 7 ,, .. | | 2 | 4 | 2 | 6 | 2 | 9 | 6 | 19 |
| ,, ,, ,, 14 ,, .. | | 1 | 4 | 2 | 6 | 2 | 9 | 5 | 19 |
| ,, ,, ,, 21 ,, .. | | 1 | 3 | 1 | 5 | 2 | 9 | 4 | 17 |
| Staphylococcal lesions in survivors | None | 1 | 1 | | 2 | | 6 | 1 | 9 |
| | Slight | | 1 | 1 | | | | 1 | 1 |
| | Gross | | 1 | | 3 | 2 | 3 | 2 | 7 |

these conditions the cephalosporin not only increased the length of life but enabled some of the animals to survive the infection. The size of the inoculum of staphylococcus seemed to make some difference to the numbers surviving.

Nevertheless, the results were disappointing, for it is probable that an inhibitory level of cephalosporin was maintained in the blood for some five and a half days, and the defences of the animals might have been expected to deal with the cocci in that time, even if the drug was only present in bacteriostatic concentrations. Only with the small inoculum of staphylococci did the defences seem adequate.

TABLE VI

COMPARISON OF THE ANTISTAPHYLOCOCCAL ACTION OF CEPHALOSPORIN $P_1$, AUREOMYCIN, TERRAMYCIN, AND PENICILLIN *in vivo*

Cephalosporin, aureomycin, and terramycin were given orally and penicillin subcutaneously. The dose shown was repeated 12-hourly for 4½ days. LD50 was at a dilution of about 1 : 10,000,000

| Drug and dose: | Control | Cephalosporin (mg.) | | Aureomycin (mg.) | | | Terramycin (mg.) | | | Penicillin 17,000 units ≡ 10 mg. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 5 | 10 | 1 | 2.5 | 5 | 1 | 2.5 | 5 | |
| A. Dilution of culture 1 : 100,000 | | | | | | | | | | |
| Mice alive at start .. | 10 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, after 24 hours | 2 | 1 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, ,, 48 ,, | 0 | 1 | 2 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, ,, 3 days | | 1 | 2 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, ,, 4 ,, | | 1 | 2 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, ,, 5 ,, | | 1 | 2 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, ,, 6 ,, | | 1 | 2 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, ,, 7 ,, | | 1 | 2 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, ,, 14 ,, | | 1 | 2 | 5 | 5 | 5 | 5 | 4 | 5 | 5 |
| ,, ,, ,, 21 ,, | | 1 | 2 | 5 | 5 | 5 | 5 | 4 | 5 | 5 |
| Staphylococcal lesions in survivors: None | | | 1 | 3 | 5 | 5 | 5 | 3 | 4 | 5 |
| Staphylococcal lesions in survivors: Slight | | | | | | | | | 1 | |
| Staphylococcal lesions in survivors: Gross | | 1 | 1 | 2 | | | | 1 | | |
| B. Dilution of culture 1 : 1,000 | | | | | | | | | | |
| Mice alive at start .. | 10 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, after 24 hours | 2 | 4 | 2 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| ,, ,, ,, 48 ,, | 0 | 3 | 0 | 5 | 5 | 5 | 5 | 4 | 5 | 5 |
| ,, ,, ,, 3 days | | 3 | | 5 | 5 | 5 | 5 | 4 | 5 | 5 |
| ,, ,, ,, 4 ,, | | 3 | | 5 | 5 | 5 | 5 | 4 | 5 | 5 |
| ,, ,, ,, 5 ,, | | 3 | | 5 | 5 | 5 | 5 | 4 | 5 | 5 |
| ,, ,, ,, 6 ,, | | 3 | | 5 | 5 | 5 | 5 | 4 | 5 | 5 |
| ,, ,, ,, 7 ,, | | 3 | | 5 | 5 | 5 | 5 | 4 | 5 | 5 |
| ,, ,, ,, 14 ,, | | 3 | | 5 | 5 | 5 | 4 | 4 | 5 | 5 |
| ,, ,, ,, 21 ,, | | 1 | | 5 | 5 | 5 | 1 | 4 | 5 | 5 |
| Staphylococcal lesions in survivors: None | | | | 1 | 4 | 2 | | | 1 | 4 |
| Staphylococcal lesions in survivors: Slight | | 1 | | 3 | 1 | 1 | | | 1 | 1 |
| Staphylococcal lesions in survivors: Gross | | | | 1 | | 2 | 1 | 4 | 3 | |

As aureomycin and terramycin, both drugs which are stated to be bacteriostatic *in vitro* at concentrations likely to be met with in the body, are being successfully used clinically for the treatment of staphylococcal infections resistant to penicillin, it was desirable to see how these drugs performed under the experimental conditions in which cephalosporin had been examined. An experiment was therefore done in which the effects of various doses of the three drugs given by stomach tube in 0.6 ml. water were compared. Two sizes of inoculum of the staphylococcus were used, and as before the injections were started one hour after infection. In addition, penicillin was administered subcutaneously to two groups of mice, large doses

being given, as there were 12-hour intervals between injections. Table VI records the results.

It is clear that both aureomycin and terramycin performed much better under these conditions than did cephalosporin.

*Development of resistance* in vivo

It will be recalled that resistance of the staphylococcus to cephalosporin was readily induced by suitable culture *in vitro*. It was possible therefore that the failure of cephalosporin in the protection tests was connected with a similar induction of resistance.

The sensitivity of a staphylococcus recovered from the blood of a mouse which died two and a half weeks after infection, and which had been treated by 5 mg. of cephalosporin by mouth every 12 hours for $4\frac{1}{2}$ days, was compared with that of the culture used to infect the mouse. The two strains were equally sensitive.

The possibility remains that the organism became resistant during treatment but in the time that elapsed between the cessation of treatment and death again became sensitive. This possibility was not tested.

## Discussion

The administration of penicillin has always been guided by the idea that the dose necessary, that is the level to be attained in the blood, bears a close relationship to the sensitivity *in vitro* of the organism causing the disease being treated. That this view holds good for penicillin has recently been reaffirmed by the extensive experimental results of Eagle, Fleischman, and Musselman (1950).

Evidence is accumulating, however, that it is not always possible to forecast the effect of an antibiotic *in vivo* from its performance *in vitro*. Thus helvolic acid, which inhibited the growth of the staphylococcus *in vitro* at considerable dilutions, was relatively ineffective *in vivo* against experimental disease, even though doses could be administered which were sufficient to confer on the blood strong inhibitory powers against the staphylococcus. The reason for this lack of therapeutic power was not elucidated, but at the time it was thought that it might be due to the fact that, unlike penicillin, the drug was bacteriostatic and not bactericidal. However, since those experiments were done aureomycin and terramycin, two drugs that are said to be bacteriostatic at concentrations likely to be met with *in vivo*, have been shown to be effective against both experimental infection in animals and natural infection in man.

Like helvolic acid, to which it has a number of chemical and biological points of similarity, cephalosporin $P_1$ has been found relatively ineffective *in vivo*, though strongly bacteriostatic *in vitro*, and even bactericidal at concentrations greater than 1 : 200,000. Cephalosporin $P_1$ is less toxic than aureomycin or terramycin when given intravenously (Harned, Cunningham, Clark, Cosgrove, Hine, McCauley, Stokey, Vessey, Yuda, and SubbaRow, 1948 ; P'an, Scaduto, and Cullen, 1950 ; Schoenbach, Bryer, and Long, 1950) ; sufficient can be given to maintain a concentration in the blood which strongly inhibits the infecting organism ; and it has much the same degree of antibacterial activity *in vitro* as aureomycin and terramycin. Nevertheless, *in vivo* it is greatly inferior to either.

The poor results given by cephalosporin $P_1$ *in vivo* do not appear to be determined by the development of resistance of the infecting organism, though this readily occurs *in vitro*, and for the present no satisfactory reason for its failure can be offered.

Just as there is a lack of correlation between the performance of cephalosporin *in vitro* and *in vivo*, so there is a similar though converse lack with aureomycin. This drug has been shown by Klein, Schorr, Tashman, and Hunt (1950) to be more active *in vivo* than its activity *in vitro* would give reason to suspect, especially when one considers the very considerable depression of its activity by serum *in vitro*. The work on aureomycin recorded in this paper accords with that of Klein *et al.* It is clear that there is something yet to be learnt about the exact mode of behaviour of cephalosporin $P_1$ and aureomycin *in vivo*. Perhaps the observations of Bigger and Ware (1950) on a factor in Lemco which makes sulphathiazole bactericidal may eventually throw some light on this matter. It may be, also, that there is some confusion in the use of the terms "bacteriostatic" and "bactericidal." It appears that aureomycin, like a number of other antibiotics, kills a very considerable proportion of the population of a sensitive species *in vitro* (Spicer, 1950), though not all. If it acts in the same way *in vivo* it would as a consequence cause a great diminution in the number of bacteria present in a lesion though not killing them all; that is, its action *in vivo* would be essentially "bactericidal." However, further work is required on the mode of action of these substances *in vivo*.

At the present time an antibiotic has to have very particular powers to compete with those already introduced into medicine, and it might be reasoned that cephalosporin $P_1$, even if fully effective against staphylococcal infection in mice, would not have had much use owing to the limited range of bacteria against which it is effective. Such a substance, however, with limited range would be very useful if it did not interfere with the growth of intestinal organisms or cause the side-effects such as nausea, vomiting, and diarrhoea which may result from the administration of aureomycin and terramycin, and especially if its range included penicillin-resistant staphylococci. It is perhaps a measure of progress in the treatment of bacterial disease that little more than 10 years ago cephalosporin $P_1$ would have been considered a substance of outstanding qualities.

## Summary

*In vitro* cephalosporin $P_1$ inhibits the growth of staphylococci, corynebacteria and *Cl. tetani* at considerable dilution. It has little effect on streptococci, Gram-negative organisms, or tubercle bacilli. Penicillin-resistant staphylococci are sensitive to the drug; serum reduces but does not abolish its action. By suitable repeated culture staphylococci are easily made resistant to its action *in vitro*. In concentrations of more than 1 : 200,000 cephalosporin $P_1$ is bactericidal, in weaker concentrations it is bacteriostatic. It is more active against the staphylococcus than helvolic acid, and has the same order of activity as aureomycin and terramycin.

Given intravenously, cephalosporin $P_1$ is less toxic than aureomycin or terramycin, the LD50 for 20 g. mice being between 500 and 750 mg./kg. No gross or microscopic lesions were found *post mortem* in mice given 5 mg. 12-hourly by mouth for 5½ days. The drug did not affect the blood pressure of a decerebrate cat, but did modify the contractions of isolated guinea-pig uterine muscle. It is well absorbed

both when given subcutaneously and when given by mouth. After oral administration serum levels are attained and maintained which are greater than those necessary to inhibit the staphylococcus *in vitro*. The drug disappears from the blood rather quickly, although little is excreted in either urine or bile.

Mouse protection experiments show that the drug has some protective action against staphylococcal infections, but that this action is much weaker than that of either aureomycin or terramycin.

No evidence is available to explain this lack of antistaphylococcal activity *in vivo* compared with the high activity *in vitro*.

We are indebted to the Medical Research Council for grants towards technical assistance and materials used in this work. The cephalosporin $P_1$ was brewed and partially purified at the Antibiotics Research Station of the Medical Research Council at Clevedon. The final preparation was done by Dr. H. S. Burton. We have received invaluable technical help from Mr. J. Kent and Miss M. Lancaster. We are indebted to Dr. M. A. Jennings, Dr. E. P. Abraham, and Dr. N. G. Heatley for help in the preparation of this paper.

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*Note added in proof:* Just before proofs of this paper were received we became aware of the fact that the aureomycin used in these experiments was not pure. It was taken from capsules made for oral administration to man, and it appears that in these it is mixed with some other material. The same is probably true of the terramycin used. A capsule that contains 250 mg. of aureomycin contains about 130 mg. of other solids. This fact makes no difference to the arguments in this paper, but it does mean that pure aureomycin and terramycin hydrochlorides are more active against the staphylococcus than appears from the figures given, and that the doses administered in the protection experiments were smaller than was supposed.

*Brit. J. Pharmacol.* (1951), **6**, 445.

# PROLONGATION OF ACTION OF CHORIONIC GONADOTROPHIN

BY

K. DIDCOCK, J. M. ROBSON, AND A. A. SHARAF

*From the Department of Pharmacology, Guy's Hospital Medical School*

(Received May 3, 1951)

It is frequently of advantage to prolong the duration of action of a drug and thus to decrease the number of administrations necessary to produce the required therapeutic effect. This has been successfully accomplished with steroid hormones, e.g., by the administration of suitable esters or by the implantation of tablets. Comparatively limited progress has attended the attempt to extend the period of action of protein-like substances. Combination of insulin with protamine or other proteins will prolong its action to some 24 hours, but attempts to extend this period still further by the use of implants have not been particularly successful (Gilliland and Martin, 1951). Posterior pituitary hormones have been administered in oil and their period of action thus somewhat extended, but to the best of our knowledge no method of prolonging the action of pituitary hormones or of gonadotrophin from other sources by the use of implants has so far been devised.

In the present paper a method is described of making implants of urinary gonadotrophin which have a prolonged effect.

## METHODS

A preparation of chorionic gonadotrophin with an activity of 446 i.u. per mg. was used in all the experiments. The implants were prepared as follows: The gonadotrophin was mixed intimately with three times its weight of magnesium monostearate* and the mixture compressed to an implant in a tableting machine.

The duration of action of such implants was investigated by determining their effect in immature mice. Female albino mice, from a strain obtained from one dealer and bred in the laboratory, were used. These mice become sexually mature in about seven weeks. In all experiments the effects of implants and of injections containing the same amount of hormone were compared on separate groups of five mice, obtained by suitably dividing litter mates according to weight. Treatment was started when the mice were about three weeks old and weighed around 10 g.

The implants (weighing 1 mg.) were inserted subcutaneously in the upper dorsal region, under ether anaesthesia. For injection, the gonadotrophin was dissolved in saline and given either as a single injection of 0.2 ml. or in six injections, each of 0.1 ml., on the morning and evening of three consecutive days. The total dose of gonadotrophin per mouse was always 0.25 mg. of the preparation ($\equiv$111.5 i.u.). Vaginal smears were taken daily in some groups and the intensity of the vaginal effect evaluated in a semi-quantitative manner, according to the method of Robson (1938). The mice were killed after intervals of 5, 10, 15 or 20 days from the beginning of the experiment. *Post mortem* the ovaries and uterus were dissected out and weighed on

* Obtained from Morson Thomas and Son, Ltd., London.

a torsion balance after being roughly dried on filter paper. In addition the number of blood spots in the ovaries was noted, as was the degree of follicular development.

The implants were recovered from all the animals *post mortem*, and implants from any particular group of five animals were pooled, dried overnight in a vacuum desiccator over phosphorus pentoxide, and the gonadotrophin extracted by grinding several times with about 1 ml. amounts of cold water which were pooled. The assay was performed on immature female rats in comparison with the actual preparation used to make up the implants. The rats were injected on the morning of five consecutive days, and killed on the sixth day; the assay was based on the ovarian weight.

## RESULTS

The results of all the experiments on immature mice are shown in Table I. Each figure in the Table represents the average for five mice. The figures under "Blood Spots" are the average numbers per ovary.

TABLE I

Showing the effects of chorionic gonadotrophin, administered as a single injection, in six injections, or as an implant, on the uterus and ovaries of immature mice at various periods after the administration. Each mouse received 0.25 mg. of a preparation containing 446 i.u. per mg. and every figure represents the average result for five mice. Results bracketed together were obtained on balanced groups of litter mates. The numbers under Ut. and Ov. represent the average weights of the uterus and ovaries in mg. The results under BS represent the average number of blood spots per ovary

| Method of administration | Interval between first administration and *post mortem* (days) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | | | 10 | | | 15 | | | 20 | | |
| | Ut. | Ov. | BS | Ut. | Ov. | BS | Ut. | Ov. | BS | Ut. | Ov. | BS |
| Single injection | 22.6 | 8.25 | 0.75 | 35.1 | 15.3 | 0.3 | | | | | | |
| Implantation | 88.0 | 12.5 | 1.6 | 49.2 | 16.9 | 2.75 | | | | | | |
| | 70.0 | 18.9 | 2.5 | 50.0 | 15.1 | 2.4 | 35.6 | 10.2 | 1.6 | 55.6 | 15.9 | 0.0 |
| Six injections | 57.3 | 14.0 | 1.0 | 24.7 | 13.2 | 0.5 | 13.2 | 6.1 | 0.0 | 11.2 | 6.6 | 0.0 |

It will be seen from both the uterine and ovarian weights that effects from the implants were still evident 20 days after their insertion. The morphological effects on the ovaries were in general agreement with this conclusion: up to 15 days there is a striking difference between the number of blood spots in the animals receiving the implants and in those receiving the same dose of gonadotrophin by injection over three days. The effects on follicular growth, not shown in the Table, were also similar, and there was, indeed, a difference between the two groups 20 days after the beginning of treatment.

The effect on the vaginal smear also showed the more prolonged effect of the implants. In the group injected over three days the smears had become negative by the eighth day, whereas in the implanted group some positive effect on the vagina was still to be seen after 20 days.

These results on the effects of the implanted gonadotrophin are confirmed by the assays of the recovered implants, the results of which are shown in Fig. 1. After

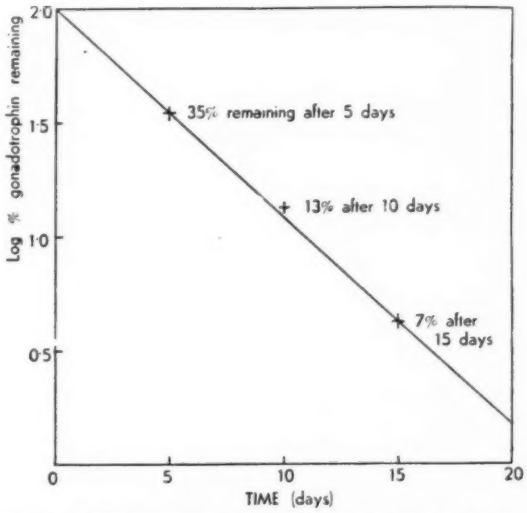


FIG. 1.—The percentage of active material (chorionic gonadotrophin) remaining in the subcutaneous implants at various periods after implantation.

five, ten, and fifteen days the implants had lost 20, 30, and 50 per cent of their weight, and 65, 87, and 93 per cent of their activity. The gonadotrophin content of the implants recovered at 20 days was not assayed, as extrapolation suggested that the amount available would be too small to estimate with any accuracy.

## DISCUSSION

The results show that the period of action of chorionic gonadotrophin is considerably extended when the hormone is administered as a compressed implant made with magnesium monostearate, a material readily available. In the particular experiments described above the implants contained one part of the active material with three parts of the excipient, and the active material was absorbed from the implant in about 20 days. Other experiments (not recorded here) suggest that the period of absorption depends on the relative proportion of active material and excipient, and that periods of action suitable for the clinical use of the material could readily be obtained by varying the relative proportions of gonadotrophin and magnesium monostearate.

Careful macroscopic examination has not revealed any reaction around the implantation sites, which have, however, not been examined histologically.

The method described here might also be suitable for extending the period of action of other protein-like substances and perhaps even of other drugs ; experiments at present in progress suggest that it may be of value in the administration of ACTH and of insulin.

## SUMMARY

A method of making implants containing chorionic gonadotrophin, by compressing a mixture of the hormone and magnesium monostearate into tablets, is described. When implanted subcutaneously into immature mice, the tablets produced an effect lasting for about 20 days. Other possible uses of the method are discussed.

We are grateful to Dr. Tindall, of Organon, Ltd., for the supply of chorionic gonadotrophin (pregnyl) used, and to the Medical Research Council for a grant (to J. M. R.) which defrayed, in part, the expenses of the investigation.

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*Brit. J. Pharmacol.* (1951), **6**, 448.

# CONVULSIVE AGENTS AND THE PHOSPHATES OF BRAIN EXAMINED *IN VITRO*

BY

G. ANGUIANO* AND H. McILWAIN

*From the Biochemical Laboratories, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E.5*

(Received April 30, 1951)

Many agents which change the functional activity of the brain *in vivo* change also its concentration of inorganic and labile phosphates (for references see McIlwain, 1950). Under suitable conditions, the concentrations of these phosphates can be maintained in sections separated from mammalian brain *in vitro* (McIlwain, Buchel, and Cheshire, 1951). Such sections therefore give a means of examining any direct actions of different agents on the metabolism of central nervous tissues without secondary—e.g., endocrine or vasomotor—effects from the rest of the body. Examination of narcotics in this way has already been reported (Buchel and McIlwain, 1950).

During convulsions caused by a variety of agents the inorganic phosphate of the brain rises and creatine phosphate and in some cases adenosine polyphosphates fall. This has been demonstrated for pentamethylene tetrazole (cardiazol ; leptazol ; metrazol) in dogs (Stone, Webster, and Gurdjian, 1945) and cats (Klein and Olsen, 1947) ; for electrical stimulation in cats (Klein and Olsen, 1947) and rats (Dawson and Richter, 1950) and also for caffeine, bromocamphor, theophylline with ethylenediamine, and nikethamide (Klein and Olsen, 1947).

In the present paper we describe our study of the phosphates of brain metabolizing *in vitro* in the presence of pentamethylenetetrazole, picrotoxin, caffeine, and strychnine and also while subject to varying potential gradients which were intended to simulate conditions during electrically induced convulsions. The effects of these agents on respiration and sometimes on glycolysis have also been observed. In most experiments glucose was used as substrate, but some with glutamic acid as substrate are included.

## EXPERIMENTAL

*For studying the action of added substances.*—The methods and salines used were based on those described by McIlwain, Buchel, and Cheshire (1951) and Buchel and McIlwain (1950). An experiment commonly employed three large manometric vessels of about 100 ml. with sidearms and centre wells, together with a thermobarometer. Two or three guinea-pigs or rats were decapitated, the cerebral cortex separated and sliced, and the slices distributed so that each experimental vessel received a representative sample of about 0.4 g. fresh weight. The vessels each contained a total of 15 ml. saline, of which 0.1–1.5 ml. was in some experiments used to contain an added substance in a

* British Council bursar.

sidearm, and mixed with the rest during an experiment. The vessels were equilibrated with $O_2$ at 37° and their $O_2$ uptake followed each 5 minutes during the experimental period, commonly (see Tables) of 30 minutes.

At the end of this time the contents of the flasks were tipped one at a time into a porcelain filter funnel with a perforated disc, so collecting the slices, which were allowed to drain for about 10 seconds. The funnel was used as a sieve, without any filtering medium. The slices were then picked up with a mounted, bent wire, drained for a few seconds on the side of the funnel and dropped into 1.5 ml. of 10 per cent (w/v) trichloroacetic acid in a homogenizer tube already cooled in ice and weighed. The tissue was immediately homogenized and later the tube was wiped dry outside and reweighed to give the wet weight of tissue used in the experiment. Meanwhile the contents of the second and third flasks were treated in the same way. As the tissue was weighed at the end of the experiment, small detached fragments of the slices could be ignored and allowed to pass the filter. On the other hand, the tissue is known to swell during such experiments; this was allowed for as described by McIlwain, Buchel, and Cheshire (1951). Inorganic and creatine phosphates were then determined in the trichloroacetic filtrate after Ca-ethanol separation, also as described by McIlwain, Buchel, and Cheshire (1951).

*For studying the action of electrical changes.*—The methods of McIlwain and Gore (1951) were used. These depended on passing varying currents (described in the Table concerned) between electrodes in the above media close to slices of brain cortex. Respiration was measured concomitantly. At the end of the experiment the slices were removed individually and rapidly from their holders and extracted as described above.

## RESULTS

Large differences have been found in the biochemical effects of different convulsive agents on separated cerebral cortex. Two extreme cases are quoted in detail in Table I.

TABLE I

PENTAMETHYLENE TETRAZOLE AND ELECTRICAL STIMULATION ON PHOSPHATES AND RESPIRATION OF GUINEA-PIG CEREBRAL CORTEX

All experiments quoted were carried out at 37° in glycylglycine-buffered medium with glucose as substrate, and similar results were also obtained in bicarbonate-buffered media. Tissue-wt./ml. = 20–25 mg. Electrical stimulation with a.c. at 2.9V in electrodes D and vessels A′ of McIlwain (1951)

| Measurements | Pentamethylene tetrazole | | | Electrical stimulation | |
|---|---|---|---|---|---|
| | Control | $10^{-3}$ M | $10^{-2}$ M | Control | Stimulated |
| $O_2$ uptake at 10 min. ($\mu$ mol./g.) | 14.6 | 14.5 | 14.4 | 9 | 17 |
| $O_2$ uptake at 20 min. ($\mu$ mol./g.) | 25.8 | 25.6 | 24.8 | 20 | 36 |
| $O_2$ uptake at 30 min. ($\mu$ mol./g.) | 37 | 36.8 | 36.1 | 32 | 52 |
| Final *p*H .. .. .. .. | 7.3 | 7.3 | 7.3 | 7.2 | 7.2 |
| Inorganic P ($\mu$ mol./g.) .. | 3.58 | 3.55 | 3.50 | 4.10 | 4.95 |
| Creatine phosphate ($\mu$ mol./g.) | 1.38 | 1.48 | 1.38 | 1.16 | 0.27 |

*Pentamethylene tetrazole* was found to have remarkably little effect on the phosphocreatine or inorganic phosphate concentration, even when examined in higher concentrations and after longer periods of action than are effective *in vivo*. The convulsive dose in man or cats would give a maximum concentration of the order

of $10^{-4}$ M, if it was uniformly distributed, or perhaps $10^{-3}$ M if it was in the central nervous system only. Concentrations of $10^{-4}$ to $10^{-2}$ M were examined *in vitro* with negative results. Respiration also was unchanged; *in vivo*, cerebral respiration increases during convulsions. Both glucose and glutamate (Table II) were used as substrates *in vitro*.

*Electrical stimulation in vitro* was found to have major effects on phosphates. Table I shows that it could lead to loss of three-quarters of the creatine phosphate, with an approximately equivalent increase in inorganic phosphate. The electrical stimulation used was not greatly above the threshold for the metabolic response. This had been found to be of the order of 1.5 V for sine-wave a.c. currents of 50 cyc./sec. (McIlwain, 1951), with cortex slices in the present apparatus. The experiments of Tables I and II employed currents of these characteristics at 2.7 and 2.9 V. This voltage is on the lower range of those found necessary in stimulating exposed cortex or in stimulating various parts of the brain with electrodes buried in it (for reference see McIlwain, 1951). It was much lower than the voltages used in electro-convulsive therapy, which are, however, not comparable, as they are applied to the outside of the head.

Electrical stimulation in the present experiments led also to increased cerebral respiration, which is found *in vivo* (Schmidt, Kety, and Pennes, 1945).

*Picrotoxin, strychnine, and caffeine.*—Results with these substances are quoted in Table II. Effects were never obtained comparable to those of electrical stimulation, although quite high concentrations were examined. Thus, with strychnine, Klein and Olsen (1947) found effects on the phosphates *in vivo* with quantities which, if evenly distributed, might yield $3 \times 10^{-6}$ M of the drug, or $10^{-4}$ M if it was largely in the central nervous system. Veit (1935) found about $2–4 \times 10^{-5}$ M strychnine in some parts of the central nervous system during its action in cats, dogs, and apes. The present experiments showed no effect on phosphates at $10^{-3}$ M, but a fall in labile phosphates at $10^{-2}$ M when glucose was used as substrate. The fall is however likely to be secondary to the change in respiration which is also recorded. In contrast to the effect of strychnine *in vivo*, respiration falls. This was associated with a decrease also in inorganic phosphate, whereas *in vivo* Klein and Olsen (1947) observed a large increase in inorganic phosphate to follow strychnine convulsions. No change in inorganic phosphate was found with glutamate as substrate.

With caffeine the stimulating dose would if immediately distributed give a mean body concentration of the order of $2 \times 10^{-5}$ M, and the convulsive dose found to affect phosphates, one of about $5 \times 10^{-3}$ M. We found a small but definite lowering of creatine phosphate with $10^{-2}$ M caffeine *in vitro*, which appeared to be independent of respiratory change and thus may be to some extent analogous to the change *in vivo*. It did not occur with glutamate as substrate.

The changes in phosphates reported by Klein and Olsen (1947) to occur on administering picrotoxin were not large but were given with doses equivalent to $2 \times 10^{-5}$ M if rapidly and evenly distributed, or about $10^{-3}$ M if mainly localized in the central nervous system. In the present experiments $10^{-3}$ or $10^{-2}$ M concentrations caused an appreciable lowering of phosphocreatine, which may therefore bear some relationship to the action *in vivo*. This change also occurred with glucose, but not glutamate, as substrate.

TABLE II

VARIOUS CONVULSIVE AGENTS ON PHOSPHATES AND RESPIRATION OF GUINEA-PIG CEREBRAL CORTEX

Experimental arrangement as described in Table I. The changes in italic type represent significant deviations from normal values

| Agent and molar concentration | Substrate | Respiration | | Inorganic P | | Creatine phospnate | |
|---|---|---|---|---|---|---|---|
| | | $\mu$ mol./g./hr. | % change from control | $\mu$ mol./g. | % change from control | $\mu$ mol./g. | % change from control |
| Pentamethylene tetrazole $10^{-3}$ | glucose | 74 | −0.5 | 3.55 | −1 | 1.48 | +7 |
| ,, ,, $10^{-2}$ | glucose | 72 | −2 | 3.50 | −2 | 1.38 | 0 |
| ,, ,, $10^{-3}$ | glutamate | 84 | 0 | 5.17 | −2 | 0.30 | −5 |
| ,, ,, $10^{-2}$ | glutamate | 84 | 0 | 4.95 | −2 | 0.36 | +17 |
| Picrotoxin $10^{-3}$ .. .. | glucose | 67 83 | −8, +4 | 3.54, 3.80 | *+12*, *+4* | 1.10, 0.90 | *−29*, *−18* |
| ,, $10^{-2}$ .. .. | glucose | 71 79 | −3, −3 | 3.23, 3.43 | +3, −6 | 1.16, 0.86 | −25, −23 |
| ,, $10^{-3}$ .. .. | glutamate | 84 | 0 | 5.2 | −2 | 0.33 | +4 |
| ,, $10^{-2}$ .. .. | glutamate | 84 | 0 | 5.45 | +3 | 6.30 | +3 |
| Strychnine $10^{-3}$ .. .. | glucose | 60 | *−13* | 3.13 | *−8* | 1.03 | −5 |
| ,, $10^{-2}$ .. .. | glucose | 57 | *−18* | 2.78 | *−18* | 0.62 | *−43* |
| ,, $10^{-3}$ .. .. | glutamate | 66 | *−15* | 5.6 | 0 | 0.43 | −4 |
| ,, $10^{-2}$ .. .. | glutamate | 62 | *−21* | 5.55 | −1 | 0.44 | −3 |
| Caffeine $10^{-3}$ .. .. .. | glucose | 75 | −5 | 3.45 | +10 | 0.93 | −8 |
| ,, $10^{-2}$ .. .. .. | glucose | 79 | 0 | 3.12 | −1 | 0.80 | *−20* |
| ,, $10^{-3}$ .. .. .. | glutamate | 80 | −4 | 5.35 | +2 | 0.30 | +8 |
| ,, $10^{-2}$ .. .. .. | glutamate | 80 | −4 | 5.15 | −2 | 0.30 | +7 |
| a.c. 50 cyc./sec., 2.7 V. .. | glucose | 144 | *+104* | 4.52 | *+40* | 0.52 | *−50* |

## Discussion

It is clear that whereas the biochemical events occurring in the brain during electrically induced convulsions have been imitated *in vitro* in sections of cerebral cortex, this was not so with those associated with pentamethylene tetrazole or strychnine in the present experiments. Results with picrotoxin and caffeine were not sufficiently definite. The similarity in the biochemical events produced by electrical stimulation *in vivo* and *in vitro* extends to respiration, glycolysis, and glucose utilization (McIlwain, Anguiano, and Cheshire, 1951) as well as to the present findings with phosphates. The slices are therefore not precluded by their handling and artificial situation from responding in a fashion typical of convulsive activity.

A possible reason for the differing responses shown by the slices to the agents examined is as follows. The slices appear to show no spontaneous electrical activity comparable to that shown *in vivo*. Indeed, undercutting the cortex in situations which involve much less disturbance than those of the present experiments stops spontaneous electrical activity (Dusser de Barenne and McCulloch, 1941 ; Burns, 1950). Chemical convulsants increase the electrical activity of the intact brain and may do so by lowering the threshold to spread of impulses in the brain. The chemical changes are then understandable as necessary to support the increased electrical activity (see, for example, McIlwain, 1950). However, in a preparation showing no spontaneous activity, such lowering of threshold would be less likely to bring about any increased electrical or metabolic changes. On the other hand, the preparations may, like those of Adrian (1936), Dusser de Barenne and McCulloch (1941), and Burns (1950), remain capable of responding to applied potentials, and hence of showing the metabolic changes reported above. Further examination of the chemical convulsants on separated tissue appears to require their addition during the application of electrical stimuli.

Glutamic acid is one of the few substances other than glucose which are oxidized rapidly by brain slices. The phosphates of the slices respiring with glutamate as substrate did not approximate to the quantities found *in vivo*, as was the case when glucose was used. Nevertheless it was of interest to see whether the chemical convulsants brought about any change in the phosphate levels. Table II shows that little change was caused. This is more likely to be due to the initially low level of creatine phosphate than to any anticonvulsive action of glutamic acid. Glutamic acid is considered in detail from this point of view elsewhere.

## Summary

1. Pentamethylene tetrazole and strychnine, whose convulsant action *in vivo* is associated with increased cerebral inorganic phosphate and decreased creatine phosphate, did not affect the phosphates of cerebral cortex slices *in vitro* when added in amounts comparable to those effective in intact animals. Higher concentrations of strychnine altered phosphates, but in a manner suggesting the change to be secondary to a small change in respiration.

2. Applied potential gradients, simulating those causing convulsions, caused changes in the phosphates and in other biochemical characteristics which were closely analogous to those occurring in the brain *in situ* during convulsions.

3. Caffeine and picrotoxin caused relatively small effects on the phosphates *in vitro*, but these may bear some relation to their action *in vivo*.

4. The present findings indicate that the changes in phosphates, respiration, and glycolysis caused in the brain by applied potentials are not mediated by vasomotor or endocrine changes, and they enable suggestions to be made regarding the mode of action of the chemical convulsants studied.

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# STUDIES IN THE CHEMOTHERAPY OF TUBERCULOSIS: PART VII. THE OXIDATION AND REDUCTION PRODUCTS OF THIOSEMICARBAZONES

BY

E. HOGGARTH AND A. R. MARTIN

*From Imperial Chemical Industries Limited, Hexagon House, Blackley, Manchester*

(Received April 27, 1951)

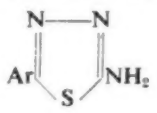
A degree of antituberculous "activity" in experimental animals has been detected with certain benzaldehyde thiosemicarbazones (I) by a number of workers (Domagk, Behnisch, Mietzsch, and Schmidt, 1946; Hoggarth, Martin, Storey, and Young, 1949). One compound of this type (*p*-acetylaminobenzaldehyde thiosemicarbazone) has had extensive clinical trials in Germany, and these trials have been critically reviewed by Hinshaw and McDermott (1950). In man, therapeutic effect is accompanied by a considerable risk of toxic side-effects, and we have made attempts to improve upon the activity and reduce the toxicity of these drugs by chemical modification of them.

TABLE I

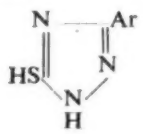
ANTITUBERCULOUS TESTS ON SOME 2-AMINO-5-PHENYL-1:3:4-THIADIAZOLES (A) AND 3-PHENYL-1:2:4-TRIAZOLE-5-THIOLS (B)

Doses given twice daily by syringe and catheter

Compounds of the forms:




(A)




(B)

| No. | Form | Ar | Dose (mg. per 20 g. mouse) | Increased mean survival time (days) | Increase required for significance (days) |
|---|---|---|---|---|---|
| 6252 | A | phenyl | 0.25 | −0.4 | 1.1 |
| | | | 0.5 | −1.1 | |
| 6289 | A | *p*-anisyl | 2.0 | −0.8 | 1.1 |
| 6560 | A | *p*-nitrophenyl | 0.25 | −0.6 | 1.0 |
| | | | 0.5 | −0.2 | |
| 6794 | B | phenyl | 10.0 | −0.9 | 1.6 |
| 6719 | B | *p*-anisyl | 2.0 | −0.6 | 1.4 |
| | | | 4.0 | −0.1 | |
| 7262 | B | *p*-chlorophenyl | 8.0 | 0.1 | 1.6 |

## Experimental Methods

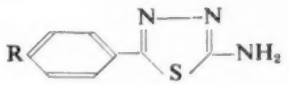
The test method consisted of the infection of mice by the intravenous route, and their treatment with drugs administered twice daily by syringe and catheter or by mixing with the food, at doses ranging downwards from the maximum tolerated ; the method has already been described in detail (Martin, 1946 ; Hoggarth and Martin, 1948). Drugs were given for a period of about two weeks.

## Results

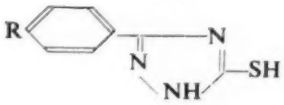
It is characteristic of aldehyde thiosemicarbazones as a class that, under mild oxidizing conditions, cyclization takes place, with formation either of 2-amino-5-phenyl-1 : 3 : 4-thiadiazoles (II), or of 3-phenyl-1 : 2 : 4-triazole-5-thiols (III) (Young and Eyre, 1901 ; De and Roy-Choudhury, 1928). We have developed improved methods for the preparation of these cyclized compounds (Hoggarth, 1949) and have examined in tuberculous mice the examples listed in Table I. During the course of the chemical investigations, a number of related cyclic structures were synthesized, and some twenty compounds belonging to the following closely related classes were also examined. No compound showed any activity.

2-alkylamino-5-phenyl-1 : 3 : 4-thiadiazoles
2-amino-5-phenyl-1 : 3 : 4-oxadiazoles
2-alkylamino-5-phenyl-1 : 3 : 4-oxadiazoles
4-alkyl-3-phenyl-4 : 1 : 2-triazole-5-thiols
5-hydroxy-3-phenyl-1 : 2 : 4-triazoles
5-amino-3-phenyl-1 : 2 : 4-triazoles
4 : 5-diamino-3-phenyl-4 : 1 : 2-triazoles

(I)

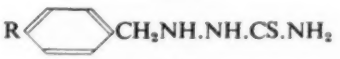



(II)




(III)

(IV)




(V)

(VI)

Whilst the oxidation of thiosemicarbazones with cold " perhydrol " in acetic acid was being studied, a group of novel sulphonic acids (IV) was isolated (Hoggarth, 1951, patent protection pending). Examination of the sulphonic acids listed in Table II showed that certain of these compounds had " activity " about equal to that of the corresponding thiosemicarbazones. We have also shown (Hoggarth and Young, 1950) that mild reduction of thiosemicarbazones gives 1-benzylthiosemicarbazides (V). These compounds were also examined and found to show

TABLE II

ANTITUBERCULOUS TESTS ON SOME 1-AMINO-4-PHENYL-2 : 3-DIAZABUTA-1 : 3-DIENE-1-SULPHONIC ACIDS

Doses given mixed with powdered food; the dose quoted is the estimated daily intake per 20 g. mouse per day

Compounds of the form: $ArCH:N.N:C(SO_3H).NH_2$

| No. | Ar | Dose (mg.) | Increased mean survival time (days) | Increase required for significance (days) |
|---|---|---|---|---|
| 8243 | phenyl | 20.0 | 3.4 | 1.2 |
| 8244 | *p*-anisyl | 20.0 | −0.2 | 1.2 |
| 9111 | *p*-ethylsulphonylphenyl | 16.0 | 10.4 | 1.7 |
| 9669 | *p*-acetylaminophenyl | 16.0 | 4.4 | 1.3 |
| 10487 | *p*-tolyl | 20.0 | −0.2 | |
| | | 10.0 | −0.1 | 2.2 |
| | | 4.0 | −0.4 | |
| 10488 | *p*-chlorophenyl | 10.0 | 1.8 | 1.8 |
| | | 4.0 | −1.7 | |

"activity" in general about equal to that of the parent thiosemicarbazones (Table III). No activity was found among a group of closely related compounds which included the types listed below. The first two classes are positional isomers of the reduction products of thiosemicarbazones, and the second two classes are derived by reduction of substituted benzaldehyde semicarbazones and acetophenone thiosemicarbazones respectively.

2-benzylthiosemicarbazides

4-benzylthiosemicarbazides

1-benzylsemicarbazides

1 : 1′-phenylethylthiosemicarbazides

The previously mentioned sulphonic acids (IV) are readily hydrolysed and yield, according to the conditions, either benzaldehyde semicarbazones, which have been found to be inactive (Hoggarth, Martin, Storey, and Young, 1949), or benzaldehyde azines (VI). The results of the examination of some fifteen azines and related derivatives of hydrazine were also negative except for slight, though reproducible, activity with a dibenzoylhydrazine (1-*p*-aminobenzoyl-2-benzoyl-hydrazine, No. 8137). The hydrazine derivatives examined in this connexion were of the classes listed below. The last group of compounds arose from the first group by a facile cyclization.

1 : 2-dibenzoylhydrazines

benzhydrazides

2 : 5-diphenyl-1 : 3 : 4-oxadiazoles

TABLE III

ANTITUBERCULOUS TESTS ON SOME 1-BENZYLTHIOSEMICARBAZIDES

Doses given twice daily by syringe and catheter or mixed with powdered food. In the latter case the dose quoted is the estimated daily intake per 20 g. mouse

Compounds of the form: $ArCH_2NH.NH.CS.NH_2$

| No. | Ar | Dose (mg. per 20 g. mouse) | Increased mean survival time (days) | Increase required for significance (days) |
|---|---|---|---|---|
| 7314 | phenyl | 0.5 | 0.9 | 1.6 |
| | | 1.0 | −0.1 | 1.6 |
| | | 0.4 mg. per day in food | 1.3 | 1.3 |
| 7316 | *p*-dimethylaminophenyl | 5.0 | 4.3 | 1.6 |
| | | 5.0 | 6.4 | 1.3 |
| | | 5.0 mg. per day in food | 7.0 | 1.3 |
| 7315 | *p*-anisyl | 2.0 | 2.9 | 2.5 |
| | | 4.0 | 3.9 | 2.5 |
| | | 5.0 | 6.1 | 1.5 |
| | | 6.0 | 4.5 | 2.5 |
| | | 5.0 mg. per day in food | 8.7 | 1.7 |
| 7317 | *p*-hydroxyphenyl | 5.0 | −1.1 | 1.8 |
| | | 5.0 | −2.0 | 2.5 |
| | | 10.0 | 0.6 | 1.5 |
| | | 10.0 | 1.0 | 2.5 |
| | | 10.0 per day in food | 1.7 | 1.3 |
| 9112 | *p*-ethylsulphonylphenyl | 10.0 | 9.8 | 1.7 |

## DISCUSSION

The narrowness of the limits within which "activity" is found with compounds related to the thiosemicarbazones (which was noted in Part V of this series, Hoggarth, Martin, Storey, and Young, 1949) is again emphasized. The positive effects observed in tuberculous mice with 1-benzylthiosemicarbazides (Table III) were very similar to those previously reported for the parent thiosemicarbazones, and in view of the fact that these reduction products are very readily reoxidized to thiosemicarbazones (Hoggarth and Young, 1950) this was not surprising. It was also to be expected that the corresponding reduction products of benzaldehyde semicarbazones and acetophenone thiosemicarbazones would show no activity. The very low "activity" of 1-*p*-hydroxybenzylthiosemicarbazide (No. 7317) was, however, unexpected, since the corresponding thiosemicarbazone (No. 6082), previously examined, had shown high "activity." Behnisch, Mietzsch, and Schmidt (1950) have reported in general terms that 1-benzylthiosemicarbazides show antituberculous activity.

The results with the 1-amino-4-phenyl-2 : 3-diazabuta-1 : 3-diene sulphonic acids (Table II) do not parallel those with the parent compounds. Thus No. 8243, derived from an "inactive" thiosemicarbazone, produced a positive effect, whilst

No. 8244, which is similarly derived from an " active " parent, did not. It is possible that *in vivo* the 1-benzylthiosemicarbazides undergo oxidation to thiosemicarbazones, but it is much less likely that the sulphonic acids are reduced to the parent compounds. The sulphonic acids may therefore produce their effects by a mechanism distinct from that of the thiosemicarbazones, and the lack of parallelism in the tests with the sulphonic acids as compared with the corresponding parent compounds would not then be surprising. The oxidative cyclizations so characteristic of thiosemicarbazones do not appear to take place *in vivo*. Extracts from the blood of animals dosed with thiosemicarbazones do not show absorption bands corresponding to either of the known types of cyclization product (Spinks, 1949).

## Summary

Some sixty compounds derived from thiosemicarbazones by a variety of oxidative and reductive processes, or closely related to such compounds, were examined in tuberculous mice. Two groups of " active " compounds were found—viz., 1-benzylthiosemicarbazides and 1-amino-4-phenyl-2 : 3-diazabuta-1 : 3-diene sulphonic acids ; the activity was of the same order as that found in the parent series of thiosemicarbazones.

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*Brit. J. Pharmacol.* (1951), **6**, 459.

# ACTION OF CALABASH CURARE AND RELATED CURARIFORM SUBSTANCES ON THE CENTRAL NERVOUS SYSTEM OF THE CAT

BY

S. SALAMA* AND SAMSON WRIGHT

*From the Department of Physiology, Middlesex Hospital Medical School, London, W.1*

(Received May 5, 1951)

In a previous paper (Salama and Wright, 1950) we described the effects of *d*-tubocurarine chloride on the central nervous system; we report in this paper the results of a study of the central actions of a number of other naturally occurring alkaloids with "curariform" action. The substances examined were: (i) calabash curare; (ii) *l*-bebeerine (curine) and *d*-bebeerine; (iii) curine dimethyl ether dimethiodide; (iv) $\beta$-erythroidine and dihydro-$\beta$-erythroidine. The chemistry of these substances is fully reviewed by Craig (1948). Some relevant chemical and physiological properties are briefly summarized below.

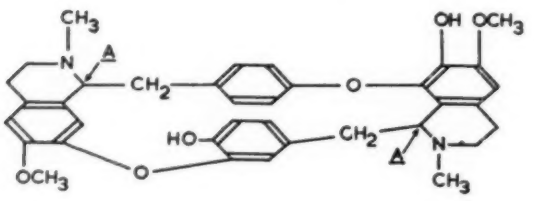
(i) *Calabash curare.*—The alkaloids isolated from calabash curare are called calabash curarines and toxiferines. The formulae of toxiferine I and calabash curarine II (which have powerful "curariform" actions) are $C_{20}H_{21}N_2^+,Cl^-$ and $C_{20}H_{23}N_2^+,Cl^-$ respectively. The non-quaternary nitrogen atom is secondary and non-basic and there is evidence for the quaternary nitrogen being in a reduced *iso*quinoline ring system and common to two rings (Karrer, 1946).

The sample used in this work was kindly provided by Dr. Harold King and was a mixture of calabash curare alkaloids. The literature on the central action of calabash curare is scanty, as earlier workers did not differentiate between the different curare alkaloids. Blume (1934) reported convulsions and other signs of central nervous stimulation in rats, mice, and guinea-pigs and to a less extent in cats after subcutaneous or intravenous administration of calabash curare.

(ii) l-*Bebeerine (curine) and* d-*bebeerine.*—The samples were kindly provided by Dr. Harold King. These tertiary bases are stereoisomeric and differ only in having opposite configurations at each of the two asymmetric centres *A* shown in the structural formula (I). The closely related bis-quaternary salt *d*-tubocurarine chloride (II, $R=R'=H$, $X=Cl$) differs in addition in probably having one dextrorotatory and one laevorotatory asymmetric centre, whereas *d*-bebeerine has both dextrorotatory (King, 1948). According to West (1937) bebeerine has a central depressant action.

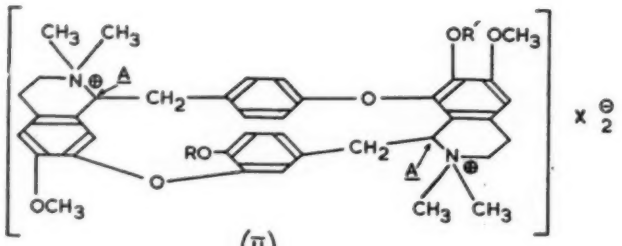
(iii) *Curine dimethyl ether dimethiodide* (II, $R=R'=CH_3$, $X=I$).—Although the term curine is the general name given by Boehm to the tertiary alkaloids which accompany the quaternary (e.g., tubocurarine) in crude curare preparations, it is now usually used as an alternative to *l*-bebeerine. The curine dimethyl ether dimethiodide is a bis-quaternary derivative of curine.

*The main results were reported to the Physiological Society (April, 1948) by S. Salama and incorporated in a Ph.D. thesis accepted by the University of London.



(I) l-Bebeerine (Curine)

A: asymmetric centres



(II)

d-Tubocurarine Chloride, R = R′ = H, X = Cl.

Curine dimethyl ether dimethiodide, R = R′ = $CH_3$, X = I.

(iv) *β-Erythroidine and dihydro-β-erythroidine.*—The active principles of *Erythrina americana* include two isomeric dextrorotatory alkaloids called α- and β-erythroidine. The latter is more readily purified and has the empirical formula $C_{16}H_{19}NO_3$; the single N atom is tertiary and common to two rings; a methoxyl group, two olefinic double bonds, a lactone ring, and an indole nucleus are also present. By catalytic hydrogenation, β-erythroidine is converted into dihydro-β-erythroidine and two tetrahydro isomers.

The erythroidines are peculiar in being tertiary nitrogen compounds with a marked peripheral paralysing action (Unna, Kniszul, and Greslin, 1944). Dihydro-β-erythroidine has a greater paralysing activity than β-erythroidine on the sciatic-gastrocnemius preparation of the cat. The peripheral paralysing activity of β-erythroidine is reduced to about a twentieth when it is converted to the quaternary methiodide. In all other bases studied the conversion of a tertiary into a quaternary compound greatly enhances the curariform action.

The samples of the erythroidine alkaloids used were kindly provided by Dr. J. W. Trevan.

## Methods

The methods employed to elicit and record the reflexes and the response of the peripheral nerve-muscle preparation were those described in the previous paper (Salama and Wright, 1950). Cats were used, usually anaesthetized with chloralose (0.06–0.08 g. per kg. body-weight). In our study of *d*-tubocurarine we found that the response of the animal to direct application of the drug to the central nervous system was the same whether the animal was anaesthetized with chloralose or decerebrated. The results to be described for all the alkaloids studied were also independent of the anaesthetic used.

Some of the alkaloids examined depressed the hind-limb reflexes. To determine whether these effects were due to an action on the central nervous system or to a peripheral action on the muscles, the ischaemic hind limb technique, described by Schweitzer and Wright (1937) was employed.

## RESULTS

### *Action of calabash curare*

*Peripheral action.*—The peripheral paralysing activity of the calabash curare preparation employed was 75 per cent that of *d*-tubocurarine when the drugs were injected intravenously in the cat, and the responses of the sciatic nerve-gastrocnemius muscles were studied. The relative activity was 50 per cent that of *d*-tubocurarine on the isolated rat nerve-diaphragm preparation.

*Central actions*

*A. Intraventricular injection.*—The effects of intraventricular injection of calabash curare in doses of 0.2–0.4 mg. (0.05–0.15 mg./kg.) into cats under chloralose anaesthesia resembled those produced by similar doses of *d*-tubocurarine injected by this route. Calabash curare produced hyper-reflexia, which was soon followed by seemingly " spontaneous " tonic and clonic movements. It also stimulated the respiration markedly, but the action on the blood pressure was generally not conspicuous.

*Skeletal muscle reflexes.*—Fig. 1 illustrates a representative experiment in which 0.2 mg. (0.055 mg./kg.) of calabash curare was injected intraventricularly. The knee jerk was immediately enhanced and a little later a shortening reaction was appended to it. After half a minute the height of the knee jerk had increased

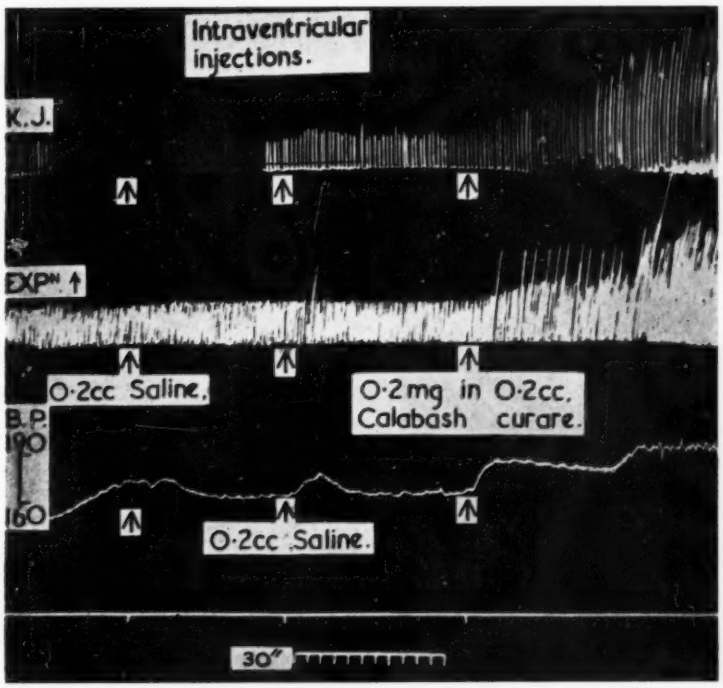



FIG. 1.—Cat, 3.6 kg., chloralose. Records from above downwards are: knee jerk; respiration (expiration upwards); carotid blood pressure; signal line; time in 30 sec. At first and second arrow, 0.2 c.c. saline injected intraventricularly. At third arrow 0.2 mg. of calabash curare in 0.2 c.c. saline injected intraventricularly.

fivefold. Within six minutes "spontaneous" movements appeared. As with *d*-tubocurarine, within one minute of the injection there was twitching of the ears and the facial muscles, followed by gross convulsive movements in the head, neck, and forelimbs. These convulsions spread quickly (in about one minute) to the hind limbs and gained in strength and frequency. In general the results were qualitatively indistinguishable from those which followed an injection of *d*-tubocurarine.

*Respiration.*—The effects of intraventricular injection of calabash curare were similar to those produced by *d*-tubocurarine. There was always a marked stimulation of respiration (Fig. 1). Sometimes the increase in rate preceded the increase in depth ; at other times, the reverse occurred. This enhancement of respiration set in before there was any significant increase in general muscular activity and still occurred after both carotid sinus regions had been denervated and both vagi cut in order to eliminate the action of the chemoreceptors.

With the onset of generalized convulsions the respiratory movements became convulsive too. The spasm of the respiratory muscles, which was only occasionally observed after injection of *d*-tubocurarine, occurred more regularly with calabash curare.

*Blood pressure.*—The vasomotor centre was less intensely stimulated by calabash curare than by *d*-tubocurarine. Thus the rise of blood pressure was small in the experiment illustrated by Fig. 1 ; this slight rise followed immediately on the injection, and was sustained. When convulsions appeared, there was a further rise of blood pressure, which may be attributed to asphyxia, as it disappeared on applying artificial respiration. Additional factors which may contribute to the onset of asphyxia are tracheobronchial obstruction and pulmonary oedema (see below).

*Glandular secretions.*—The stimulation of salivary, lacrimal, and bronchial secretions was much more marked with calabash curare than with *d*-tubocurarine. In fact, in some of the fatal cases the trachea was found after death to be blocked with thick mucous secretion and the lungs were engorged with fluid ; pressure on the abdomen in the intact animal led to the discharge of a large volume of watery fluid from the mouth.

*Eye changes.*—All the eye changes that occurred after an injection of *d*-tubocurarine were also produced by calabash curare. Thus there was a gradual increase in the size of the pupils ; there was alternating contraction and dilatation of the pupils ; the lids became retracted ; finally the eyes became widely opened and there was full mydriasis.

It can be readily shown that the *immediate* effects on the spinal reflexes produced by intraventricular injection of calabash curare are due to an action on supraspinal centres. Thus similar changes in the hind-limb reflexes, both in magnitude and in time relationship, were obtained if the spinal theca was blocked in the midthoracic region to prevent the drug from reaching the lumbar cord via the cerebrospinal fluid (Salama and Wright, 1950). The findings in this respect are similar to those with *d*-tubocurarine. The absorption of calabash curare from the ventricles into the blood is slow as judged by the absence of any depressant effect on the response of the nerve-muscle preparation by the doses employed.

*B. Effects produced by intrathecal injection.*—In order to study the *direct* effect of calabash curare on the lower spinal centres a very tight ligature was tied round the meninges and spinal cord at the level of T6 so as to abolish transmission of all nervous impulses up and down the spinal cord as well as to block the upward flow of cerebrospinal fluid to the supraspinal levels (Salama and Wright, 1950); the drug was then injected intrathecally below the block. With this technique it was found that calabash curare had a *direct* excitant effect on the spinal centres. Spinal reflexes were, however, enhanced more rapidly and to a greater extent when calabash curare was applied directly to the supraspinal than when it was applied to the spinal centres.

The minimal dose of calabash curare needed to produce significant spinal excitation by a direct action on the cord was generally twice to four times as large as that needed when the drug was applied supraspinally. The same ratio held for the dose needed to produce maximal excitation of the cord. On supraspinal application the cord effects came on almost immediately; with spinal application there was a latent period of several minutes. The direct excitant action of calabash curare on the spinal cord was, however, considerably greater than that of *d*-tubocurarine.

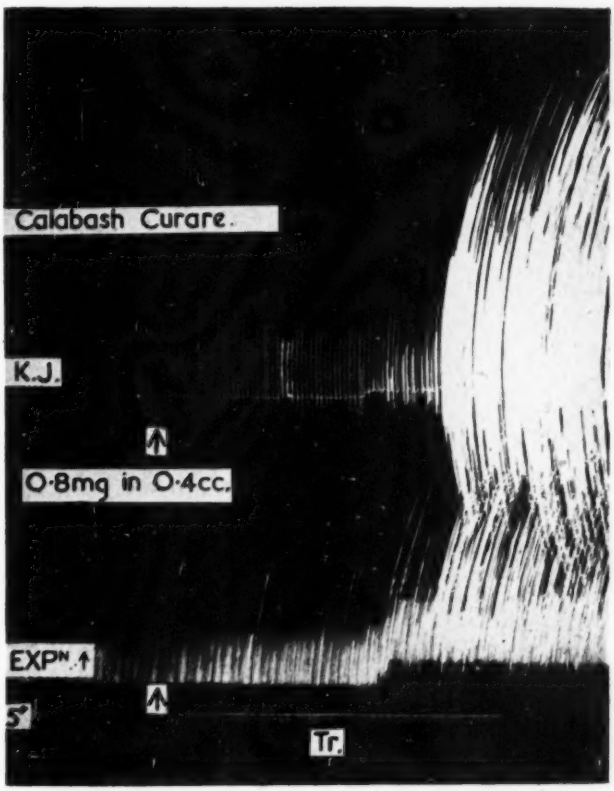



FIG. 2.—Cat, 2.5 kg., chloralose. Spinal cord transected and subarachnoid space blocked by tight ligature tied round meninges and spinal cord at level of T6. Records from above downwards are: knee jerk; respiration (expiration upwards); time in 5 sec.; signal line. At first arrow 0.8 mg. calabash curare in 0.4 c.c. saline injected intrathecally below the block. At Tr. a few drops of cerebrospinal fluid were allowed to escape in order to restore its pressure to normal.

The results of a representative experiment in which calabash curare was injected intrathecally below a complete spinal block at T6 were as follows: The dose (0.23 mg./kg.) injected was about twice as large as the average dose required to produce maximal reflex effects when given by the intraventricular route. Within three minutes of the injection, there was an increase in the knee jerk ; " spontaneous " movements appeared within 10 minutes. A response of similar magnitude only appeared as a rule after a delay of about one hour when a dose of *d*-tubocurarine three times the maximal intraventricular dose was injected intrathecally below a spinal block. After 25 minutes the convulsions were violent, but there was no change under these conditions in the blood pressure ; the eye changes and the secretory effects were also absent. Fig. 2 illustrates an experiment in which the direct excitant spinal action of calabash curare was exceptionally striking. Though the respiratory record in Fig. 2 seems to indicate that stimulation of respiration was taking place, careful inspection showed that the rhythmic respiratory movements were not changed either in rate or depth, but that the record was disturbed by the convulsive movements (owing to heightened spinal cord excitability) below the level of the block.

*Action of* l-*bebeerine and* d-*bebeerine*

The bebeerines had no central excitant or depressant actions when injected repeatedly intraventricularly or intrathecally in doses up to 10 mg./kg. body-weight. The peripheral curarizing action of *l*-bebeerine when tested on the isolated diaphragm preparation was less than 8 per cent that of *d*-tubocurarine.

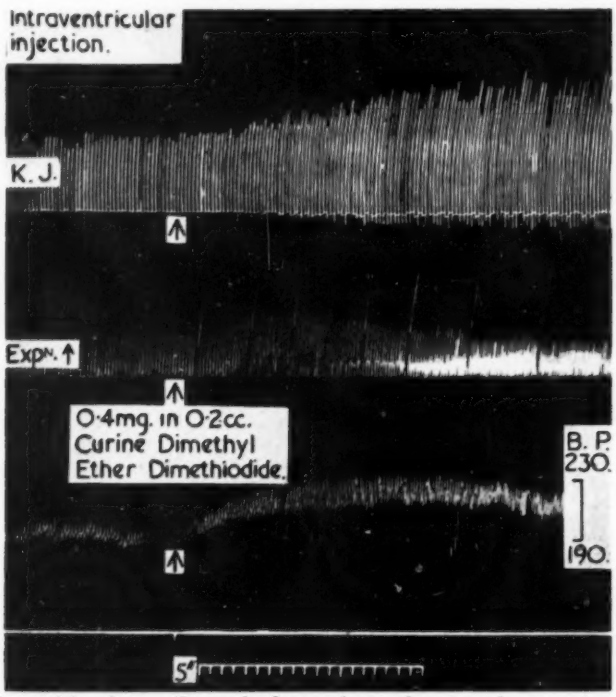
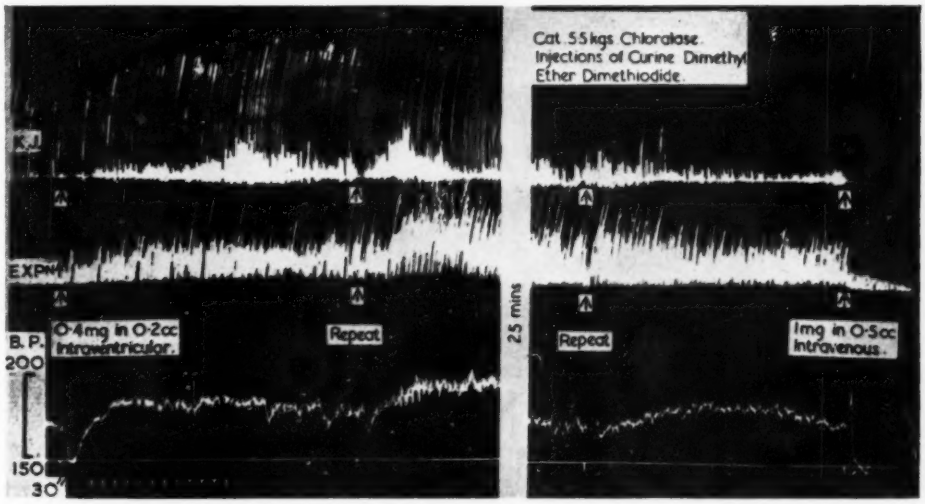



FIG. 3.—Cat, 5.5. kg., chloralose. Records from above downwards are: knee jerk; respiration (expiration upwards); blood pressure; signal line; time in 5 sec. At arrow 0.4 mg. curine dimethyl ether dimethiodide in 0.2 c.c. saline injected intraventricularly.

*Action of curine dimethyl ether dimethiodide*

When this curine compound was injected intraventricularly or intracisternally it had a central excitant action which was, however, weaker than that of *d*-tubocurarine or calabash curare. When injected intrathecally below a complete spinal block no central effects were produced. Its central excitant action is thus exerted solely on the supraspinal levels.

*A. Intraventricular injection.*—Fig. 3 illustrates the effects produced by intraventricular injection of doses of 0.4 mg. (0.07 mg./kg.) of this curine compound. Immediately after the injection, the knee jerk showed an increase in amplitude, but no " spontaneous " movements occurred during the first three minutes. A second dose of 0.4 mg. (Fig. 4A) produced a further increase in the knee jerk as well as " spontaneous " movements which were of small amplitude, but of high frequency.




A B

FIG. 4.—Cat, chloralose. Continuation of experiment in Fig. 3. (A) 3 min. after end of Fig. 3. At first and second arrows 0.4 mg. curine dimethyl ether dimethiodide injected intraventricularly. Pause of 25 min. between A and B. (B) At third arrow injection of 0.4 mg. curine dimethyl ether dimethiodide intraventricularly repeated. At fourth arrow 1 mg. of the same alkaloid injected intravenously.

After a few minutes, however, these " spontaneous " movements tended to die down, though the increase in the knee jerk persisted. A third injection of 0.4 mg. (Fig. 4A) produced another bout of " spontaneous " movements, which in their turn died down gradually over a period of 25 minutes. The knee jerk diminished as well. A fourth intraventricular injection (Fig. 4B) produced no response. An *intravenous* injection, however, of 1.0 mg. rapidly produced complete muscular paralysis (Fig. 4B).

*Respiration.*—After an initial considerable increase in depth (2.3 times), respiration soon decreased in amplitude but increased in rate. With further injections, however, the respiratory movements became involved in the general convulsions (Fig. 4A, B).

*Blood pressure.*—This showed a very slight and gradual increase, which was not sustained for more than one minute and then tended to decline gradually to its previous level.

*B. Intrathecal injection.*—2.0 Mg. doses of curine dimethyl dimethiodide were repeatedly injected intrathecally below a complete spinal block at the level of T6. There was no change in the reflexes, in the responses of the nerve-gastrocnemius preparation, in respiration or in blood pressure.

### *Action of the erythroidine alkaloids*

The erythroidines depress spinal reflexes when large doses are employed.

#### *Action of dihydro-β-erythroidine*

*Peripheral action.*—The peripheral paralysing action of dihydro-β-erythroidine on intravenous injection in the cat (nerve-gastrocnemius preparation) or on the isolated rat nerve-diaphragm was approximately 30 per cent that of *d*-tubocurarine and about the same as that of curine dimethyl ether dimethiodide.

*Central actions.*—Injected intrathecally, intracisternally, or intraventricularly in doses of less than 20 mg. (about 7 mg./kg.) dihydro-β-erythroidine produced no

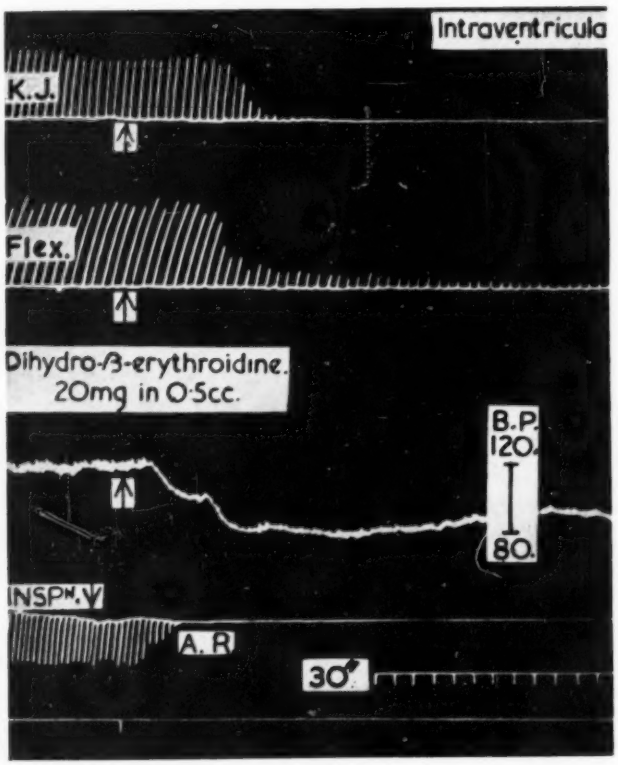



FIG. 5.—Cat, chloralose. Records from above downwards are: knee jerk (right side); flexor reflex (left side); carotid blood pressure; respiration; time in 30 sec.; signal line. At arrow 20 mg. dihydro-β-erythroidine in 0.5 c.c. saline injected intraventricularly. At A.R.: artificial respiration started.

effects. Two injections of 20 mg. intrathecally at intervals of a few minutes were likewise ineffective. Intracisternal or intraventricular injection of 20 mg., however, produced striking changes (Figs. 5 and 6). There was an immediate, moderate, but sustained fall of blood pressure, e.g., from 120 to 80 mm. Hg ; this fall is probably due to depression of the vasomotor centre. Breathing soon decreased in depth

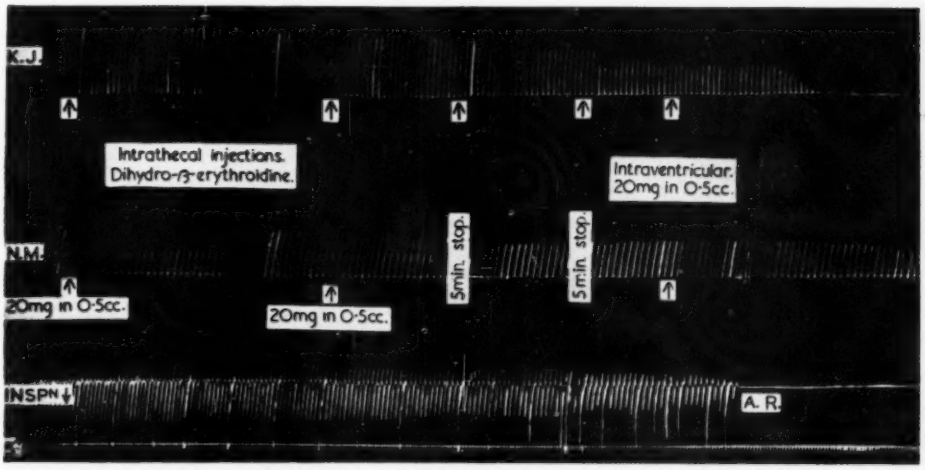



FIG. 6.—Cat, chloralose. Records from above downwards are: knee jerk (right side); contractions of gastrocnemius (left side) stimulated through its motor nerve; respiration; time in 5 sec. At the first and second arrows, 20 mg. dihydro-β-erythroidine injected intrathecally. At third arrow, 20 mg. dihydro-β-erythroidine injected intraventricularly. Drum stopped twice for periods of 5 min. At A.R.: start artificial respiration.

and finally stopped probably owing to depression of the respiratory centre. About two minutes after the injection, the spinal reflexes (knee jerk, flexor reflex) decreased rapidly in amplitude and disappeared after 3–5 minutes.

The depression of the spinal reflexes has been examined further. It is not due to asphyxia, as it occurs when the animal is maintained under artificial respiration. It is not due to ischaemia of the spinal cord, as in our experience a blood pressure level of 80 mm. Hg is adequate to maintain the normal excitability of the cord. To some extent it is due to dihydro-β-erythroidine being absorbed into the circulation and exerting a peripheral paralysing action on the muscles. The spinal reflexes were, however, depressed when the ischaemic hind-limb technique was employed in order to prevent the drug from reaching the muscles. The depression of the spinal reflexes was therefore mainly due to a central action. But, as already explained, though such depression was produced with supraspinal injection it did not occur with intrathecal injection. Dihydro-β-erythroidine must therefore produce its effects by an action on the supraspinal levels. This action may consist of depression of facilitatory neurones or stimulation of suppressor neurones. We have no evidence to enable us to decide between these two alternatives.

*Action of β-erythroidine*

*Peripheral action.*—The peripheral paralysing action of β-erythroidine is much smaller than that of its dihydro-derivative and is less than 10 per cent that of *d*-tubocurarine. Owing to shortage of material the number of experiments per-

formed was inadequate. It was found, however, that in 20 mg. doses β-erythroidine had a weak central depressant action, which was in part exerted on the spinal cord itself and partly on the supraspinal levels.

## Discussion

The main results are summarized in the Table.

1. *Calabash curare.*—Calabash curare has a marked excitatory action on the central nervous system similar to that produced by *d*-tubocurarine except in the following respects:

(i) Intraventricular administration has a smaller excitatory action on the vasomotor centre.

(ii) The enhancement of salivary, lacrimal, and bronchial secretions is more pronounced.

(iii) The direct excitatory action on the spinal cord is produced by smaller doses and after a somewhat shorter latent period than with *d*-tubocurarine.

These quantitative differences, however, do not alter the fundamental fact that calabash curare, like *d*-tubocurarine, has a strong central excitatory action and that with both alkaloids the main central action is on the supraspinal levels. The central action of calabash curare, like that of *d*-tubocurarine, is in the opposite direction to the peripheral activity.

2. *The bebeerines* (*l*- and *d*-) show no significant peripheral or central actions. The relationship between bebeerine and *d*-tubocurarine has been mentioned earlier (page 459), and while the *lack* of central action of the former would seem to be related

| Substance | Central action | Analysis of central actions | | | Relative potencies of peripheral action | |
|---|---|---|---|---|---|---|
| | | Intra-thecal | Intra-cisternal | Intra-ventricular | Curarizing the diaphragm | Curarizing intravenous cat |
| *d*-Tubocurarine .. | Strong central excitant | + | +++ | +++ | 100 | 100 |
| Calabash curare .. | Strong central excitant | ++ | +++ | +++ | 50 | 75 |
| Curine dimethyl ether dimethiodide | Weak central excitant | 0 | + | + | 30 | 30 |
| *l*-Bebeerine Cl .. | No central action | 0 | 0 | 0 | <8 | 0 |
| Dihydro-β-erythroidine | Very weak inhibition of reflexes | 0 | — | — | 30 | 30 |
| β-erythroidine .. | Very weak inhibition of reflexes | — | — | — | 4–10 | 3–6 |

+ = Central excitant action. — = Central depressant action.

in some way to its tertiary nature and the *marked* activity of the latter to the presence of quaternary groupings, it is unwise to generalize in this connexion, in view of the many factors, known and as yet unknown, which should be taken into consideration. For example, direct comparison of the activity of bebeerine with *d*-tubocurarine will serve little to relate their physiological action to chemical structure, since stereochemical factors, now realized to be so important in drug-receptor relationships, differ in these alkaloids in a way not yet absolutely determined. It is also salutary to note that the *tertiary* erythroidines possess a peripheral action similar to that of *d*-tubocurarine (see below). Presumably, even the possession of a tertiary nitrogen atom in the *correct molecular environment* can generate a peripheral activity of this type, but to generalize on the basis of the few parameters known would be dangerous.

3. *Curine dimethyl ether dimethiodide.*—This quaternary compound is stereoisomeric with *d*-tubocurarine dimethyl ether, the former being laevorotatory. This curine compound has a central excitatory and a peripheral paralysing action like that of *d*-tubocurarine but weaker. As mentioned above the tertiary base curine (*l*-bebeerine) has no central or peripheral action. The central actions of *d*-tubocurarine dimethyl ether or of *l*-tubocurarine and its dimethyl ether have not yet been examined.

4. *The erythroidines.*—These compounds contain one tertiary nitrogen atom which is common to two rings but no *iso*quinoline grouping. They seem to constitute a distinct pharmacological group. They are obtained from a specific botanical source and are not found in crude curare preparations. Though they are tertiary bases, they display a peripheral paralysing action. Unlike the natural curare alkaloids, their quaternary derivatives are less active peripherally than the tertiary compounds. Therefore their mode of action peripherally may turn out to be different in some respects from that of curare. By their central action they depress the spinal reflexes. As these effects on the nervous system are only produced when the drugs are applied in very large doses they may be non-specific in character.

## Summary

1. The actions on the central nervous system of calabash curare, *l*- and *d*-bebeerine, curine dimethyl ether dimethiodide, β-erythroidine, and dihydro-β-erythroidine were studied in cats.

2. The quaternary curare alkaloids, *d*-tubocurarine and calabash curare, and the quaternary compound curarine dimethyl ether dimethiodide have a central excitant action and a peripheral paralysing action. The central and peripheral actions of curine dimethyl ether dimethiodide are weaker than those of *d*-tubocurarine and calabash curare. The central activity of calabash curare is approximately equal to that of *d*-tubocurarine ; it has, however, a greater direct stimulating action on the spinal cord centres.

3. The bebeerines, which are tertiary curare alkaloids, are inactive centrally and have a very feeble peripheral paralysing action.

4. The erythroidines, which are tertiary compounds, have considerable peripheral paralysing activity, no central excitant action, and a weak (possibly non-specific) central depressant action.

We are greatly indebted to Dr. S. J. Holt, of the Courtauld Institute of Biochemistry, for very useful discussions of the chemical questions considered in this paper.

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# THE ANTIDIURETIC RESPONSE TO AND EXCRETION OF PITUITARY (POSTERIOR LOBE) EXTRACT IN MAN, WITH REFERENCE TO THE ACTION OF NICOTINE

BY

GEORGE P. BURN AND R. SINGH GREWAL

*From the Department of Pharmacology, University of Oxford*

(Received May 16, 1951)

The work described in this paper is a continuation of observations already made in this laboratory on the antidiuretic action of nicotine (Burn, Truelove, and Burn, 1945 ; Walker, 1948). Taylor and Walker (1951) have recently shown that the action is followed by the appearance of an antidiuretic substance in the urine of man resembling in its properties the posterior lobe hormone and not nicotine.

If a man drinks water, the onset of diuresis indicates the arrest of the normal secretion of antidiuretic hormone. When posterior lobe extract is injected intravenously at this point, inhibition of the diuresis occurs and lasts for a time depending on the amount injected. We have determined the dose-response relationship in three subjects. We have also estimated the proportion of antidiuretic hormone excreted in the urine after the intravenous injection of posterior lobe extract in varying amounts. A few experiments have been done in which the antidiuretic response to nicotine (either inhaled in tobacco smoke or injected) has been determined, and the amount of antidiuretic hormone excreted has been measured.

## METHODS

The observations were made on three male subjects in the afternoon. Each of them emptied his bladder every 15 minutes and recorded the volume. In certain experiments in which the antidiuretic effect was expected to be small, this period was reduced to 5 minutes. At the beginning of an experiment, the subject was required to show that he was not already very hydrated by producing two volumes, each of less than 25 ml. Then he drank one litre of water in 1–2 minutes. About 45 minutes later, when the volume of urine produced in the previous 15 minutes was more than 70 ml., the injection was given, or, in some experiments, the smoking was begun. The diuresis was then usually inhibited for a varying time, after which it increased to reach a second peak. The urine output was recorded until this peak was passed.

The pituitary (posterior lobe) extract (PLE) was prepared by British Drug Houses, Ltd. Nicotine was injected as a solution of nicotine hydrogen tartrate in saline. This salt contains one-third by weight of nicotine base. All injections were made intravenously. The doses were usually contained in 0.5 ml., except the large doses of PLE and nicotine, which were in 1 ml.

The method of extraction of antidiuretic hormone from urine was that described by Noble, Rinderknecht, and Williams (1939). Our practice was to reduce the volume of solvent as much as possible, using each portion twice if need be, so as to shorten the distillation.

TABLE I

TIMES TO 50 PER CENT EXCRETION DETERMINED IN CROSS-OVER TEST IN 16 RATS

| Exp. | Standard (min.) | Test (min.) | Percentage recovered |
|---|---|---|---|
| 1 | 169 | 160 | 78 |
| 2 | 133 | 128 | 87 |
| 3 | 156 | 147 | 77 |

The urine passed before injection, and that passed after, were extracted separately, giving two solutions, a blank and a test. A suitable amount of PLE was added to the blank solution to make it comparable with that present in the test solution. The blank plus the known amount of PLE then became the Standard, and these two solutions were assayed by the method of Burn (1931). All results were obtained by carrying out the full cross-over test in 16 rats.

## RESULTS

*Efficiency of extraction.*—Three experiments were performed to test the method of urine extraction. On each occasion normal urine was obtained and divided into two parts. To one part PLE was added in the proportion of 100 milliunits (mU.) to 100 ml., and both parts were then extracted. The first extract was the test solu-

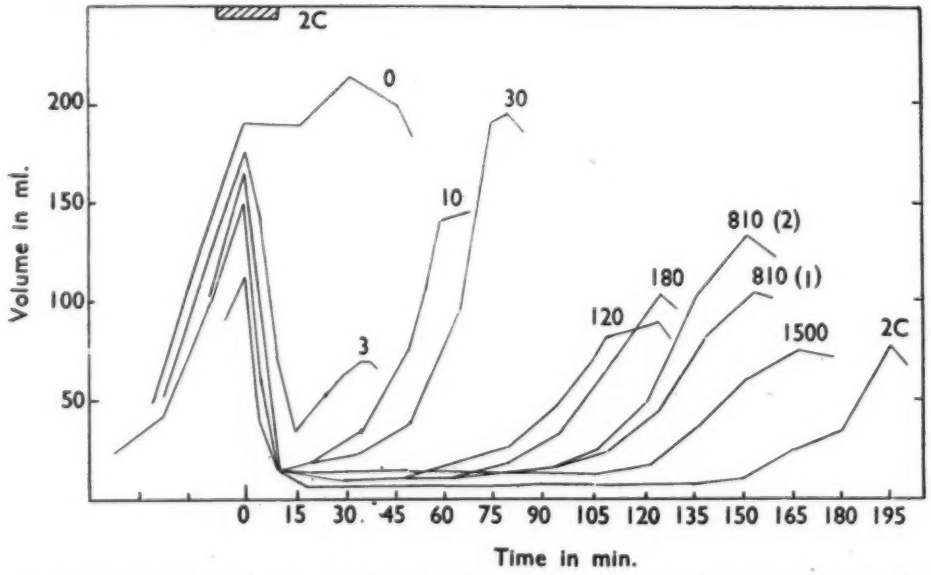


FIG. 1.—Relation between the dose of PLE and the urinary excretion. Ordinates: volume of urine in ml. per 15 min. period. Abscissae: Time in min. An intravenous injection of the dose, in mU. PLE, stated on each curve was made at zero time. The curve showing the excretion after the smoking of two cigarettes (2C) has been adjusted so that the onset of inhibition coincides with that for the other curves.

TABLE II

RELATION OF DOSE OF PLE TO PERIOD OF ANTIDIURETIC ACTION

| Dose (mU.) | Time (min.) from injection to peak of diuresis | | |
|---|---|---|---|
| | A | B | C |
| 3 | 35 | — | — |
| 10 | 70 | 68 | 67 |
| 30 | 82 | 103 | 81 |
| 120 | 128 | 113 | 98 |
| 180 | 123 | 123 | 118 |
| 180 | — | — | 113 |
| 270 | 113 | 125 | 110 |
| 405+405* | 155 | — | 123 |
| 810 | 137 | 158 | 158 |
| 810 | 153 | — | 138 |
| 1,500 | 166 | — | 150 |

* These doses were given at an interval of 15 minutes.

tion. A Standard solution was prepared by adding 100 mU. PLE to an amount of second extract corresponding to 100 ml. of urine. Thus any substances in the urine extract which might augment the effect of the antidiuretic hormone were equally present in both solutions. The results are given in Table I.

The doses of the Test solution given were chosen on the assumption that 100 per cent recovery would be obtained. Table I shows that this introduced no serious error. The mean percentage recovery was 81, a result which is in accord with those of Noble *et al.* (1939), who recovered 70–90 per cent of the original dose.

*Relation of dose of PLE to inhibition.*—We next observed the inhibition produced by injecting PLE. A series of results in one subject (A) is shown in Fig. 1 in which the curves of diuresis have been superimposed so that the time of injection is taken as zero in each experiment. For each dose the time elapsing between the injection and the peak of diuresis after the inhibition was determined. The results

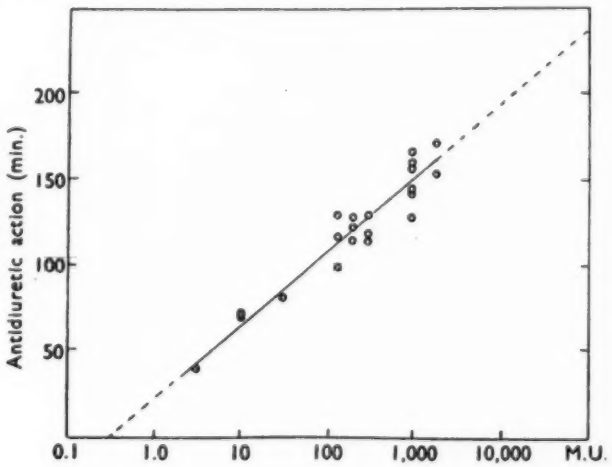


FIG. 2.—Relation between the dose of PLE and the time elapsing between the injection of the dose and the peak of diuresis which terminates the inhibition. A regression line has been drawn through the experimental points. Ordinates: Time in min. Abscissae: Dose in mU. PLE on a logarithmic scale.

are shown in Table II. The periods for all three subjects are plotted against the dose in milliunits on a logarithmic scale in Fig. 2. A regression line has been drawn through these points, and the equation of the line is

$$\text{Time in minutes} = 23.0 + 42.2 \log \text{dose}$$

where the zero of log dose is taken at 1 mU. For comparison with later theory, it is convenient to write this equation as

$$\text{Dose} = 0.285e^{0.0545t} \text{ milliunits} \quad \ldots\ldots\ldots\ldots\ldots (1)$$

an equation which relates the dose injected and the consequent average time to the peak.

*Proportion of antidiuretic hormone excreted.*—The samples of urine obtained in the above experiments were extracted, and were then examined by the rat method. The urine extract obtained after the injection until the time when inhibition was disappearing was the Test solution, and the Standard solution was prepared by adding a suitable amount of PLE to the extract of urine collected before the injection. It was found that, when the dose of PLE injected into the human subject

TABLE III

ANTIDIURETIC HORMONE IN URINE AFTER INTRAVENOUS INJECTION WITH PLE DURING WATER DIURESIS

| Dose PLE injected per subject (mU.) | No. of subjects | Quantity recovered (mU.) | Percentage recovery |
|---|---|---|---|
| 120 | 3 | 0 | 0 |
| 180 | 3 | 30 | 5.5 |
| 270 | 3 | 21 | 2.6 |
| 810* | 2 | 60 | 3.7 |
| 810 | 3 | 180 | 7.4 |
| 810 | 2 | 120 | 7.4 |
| 1,500 | 2 | 95 | 3.1 |
| | | | Mean 5.0 |

* In this experiment the dose was given in two equal parts at an interval of 15 minutes.

was 120 mU. or less, insufficient hormone appeared in the urine for it to be detected. The results are given in Table III, in which it can be seen that the mean recovery was 5 per cent, and the individual figures varied from 2.6 to 7.4 per cent.

*Action of inhaled nicotine.*—Subjects A and B smoked cigarettes on two occasions, and subjects C and D were given injections of nicotine acid tartrate, C on three occasions and D on two occasions. The duration of inhibition of diuresis was recorded and the amount of antidiuretic hormone excreted in the urine was estimated. The results are shown in Table IV. When A and B smoked cigarettes their urines were combined for the estimation of antidiuretic hormone on each occasion. In Exp. 4 of Table IV the inhibition in subject A lasted for 195 minutes. Reference to Table II shows that an inhibition of this duration would be caused by the injection of a quantity greater than 1,500 mU. PLE, which was the largest amount actually injected. In Exp. 5 of Table IV the inhibition in subject B lasted for 105 minutes, which would be caused by the injection of only 85 mU. In the

combined urines of A and B, the antidiuretic hormone found present must have come almost entirely from subject A. The amount found was 95 mU., and, on the assumption that this was 5 per cent of the amount liberated in the blood, the amount liberated in the blood of subject A was 1,900 mU.

TABLE IV

ACTION OF NICOTINE

| Exp. | Subject | Nicotine | Inhibition (min.) | Antidiuretic hormone excreted (mU.) |
|---|---|---|---|---|
| 4 | A | 2 cigarettes | 195 | 95 |
| 5 | B | 2 cigarettes | 105 | |
| 6 | A | 1 cigarette | 105 | None |
| 7 | B | 2 cigarettes | 105 | |
| 8 | C | 0.8 mg. base i.v. | 180 | 75 |
| 9 | C | 0.8 mg. base i.v. | 60 | None |
| 10 | C | 0.8 mg. base i.v. | 105 | Not determined |
| 11 | D | 1.1 mg. base i.v. | 60 | Not determined |
| 12 | D | 2.7 mg. base i.v. | 75 | Not determined |

In Exps. 6 and 7 of Table IV, the inhibition in each subject was equal to that which would be caused by 85 mU., so the total liberated in the blood of both would be 170 mU. If 5 per cent of this was excreted, the total excreted in the two urines would be 8.5 mU., which is a quantity too small to be estimated by the cross test, and in fact no antidiuretic substance was found.

*Action of injected nicotine.*—The results of injecting 0.8 mg. nicotine (base) into subject C in Exp. 8 of Table IV differed greatly from the result in Exps. 9 and 10, when the same injection was given. In Exp. 8 the period of inhibition was 180 minutes, corresponding to the release of more than 1,500 mU. In the urine 75 mU. was found, corresponding to a release of 1,500 mU. in the blood.

In Exps. 9 and 10 the injection of the same amount of nicotine both times produced a much smaller effect equivalent to that produced by the injection of 7 and 85 mU. respectively. The most likely explanation of this difference between the result of Exp. 8 and those of Exps. 9 and 10 seems to be that subject C was an apprehensive person. During Exp. 8 he experienced unpleasant effects of nausea and sweating, and when he learnt that the injection was to be repeated on the day of Exp. 9 and again in Exp. 10 he was alarmed. O'Connor and Verney (1945) observed that the antidiuretic response to an irritating faradic stimulus was irregular and that the irregularity depended on the presence of the sympathetic chain ; further, they found that the antidiuretic effect of such a stimulus was abolished by the intravenous injection of adrenaline given shortly before the stimulus was applied. Kelsall (1949) obtained a similar result. It may therefore be that the reduced effect of nicotine in subject C in Exps. 9 and 10 was due to inhibition of the action of nicotine by fear.

There is, however, a great variation in the effect of nicotine in different persons ; this is illustrated by the small effect of 1.1 mg. and 2.7 mg. in subject D.

*Effect of smoking.*—The observations made in our experiments, together with those previously recorded by Burn *et al.* (1945) and by Walker (1948), make it possible to describe the quantity of antidiuretic hormone which is released by smoking. The observations are set out in Table V, and the figures in the last column fall into two groups, namely, those in which the equivalent amount of PLE is greater than 1,000 mU., and those in which the amount ranges from 3 to 190 mU. In the experi-

TABLE V

AMOUNTS OF ANTIDIURETIC HORMONE RELEASED BY SMOKING

| Authors | Subject | No. of cigarettes | Inhibition (min.) | Equivalent amount PLE (mU.) |
|---|---|---|---|---|
| Burn *et al.* (1945) .. | L.H.T. | 3 | 210 | >1,000 |
| | L.H.T. | 3 | 120 | 190 |
| | E.M.V.W. | 3 | 210 | >1,000 |
| | E.M.V.W. | 1 | 105 | 50 |
| | J.H.B. | 1 | 195 | >1,000 |
| | T.H.C.L. | 2 | 180 | >1,000 |
| | J.R.W.G. | 2 | 195 | >1,000 |
| Walker (1948) .. .. | A (N)* | 1 | 165 | >1,000 |
| | B (N) | 1 | 165 | >1,000 |
| | C (N) | 1 | 120 | 190 |
| | D (N) | 1 | 105 | 90 |
| | E (S) | 1 | 75 | 18 |
| | F (S) | 1 | 60 | 7 |
| | G (S) | 1 | 60 | 7 |
| | H (S) | 2 | 180 | >1,000 |
| | J (S) | 2 | 120 | 190 |
| | K (N) | 2 | 90 | 40 |
| | L (S) | 2 | 90 | 40 |
| | M (S) | 2 | 60 | 7 |
| | N (S) | 2 | 45 | 3 |
| This paper .. .. | A | 2 | 195 | >1,000 |
| | A | 1 | 105 | 85 |
| | B | 2 | 105 | 85 |
| | B | 2 | 105 | 85 |

* S = smoker, N = non-smoker.

ments of the first group the subjects experienced nausea, sweating, sometimes diarrhoea, and occasionally vomiting. Sufficient nicotine was absorbed to produce toxic effects not seen when cigarettes are ordinarily smoked. In the experiments of the second group these toxic effects were inappreciable, and it is fair to take the figures of 3 to 190 mU. as indicating the amounts of antidiuretic hormone which are usually released.

The amounts vary greatly in the same individual at different times ; thus L. H. T., the first subject in Table V, smoked three cigarettes on two occasions ; on the first occasion at a time when he was not smoking, because he was in training, the amount released was more than 1,000 mU. ; on the second occasion, at a time when he was smoking about 15 cigarettes a day, the amount released was 190 mU.

So far as it is justifiable to speak of an average amount released, the mean figure is 75–100 mU. for one or two cigarettes.

## Discussion

*The rate of destruction of antidiuretic hormone.*—During the time that elapses between the injection of PLE and the subsequent peak of diuresis which marks the end of the antidiuretic effect, the hormone is destroyed. The equation of the regression line (1) shows that the logarithm of the fraction of the injected dose that is left at any time is proportional to the time that has elapsed. A simple explanation of this is that the rate of destruction of antidiuretic hormone (ADH) is proportional to the concentration. If this is true, then, when the urinary flow increases, it should do so at an exponential rate.

Now, in the experiments described, it is seen that even with the largest doses of PLE, or the most prolonged inhibitions with smoking or nicotine, the urinary output never fell to zero. There was always some excretion, amounting to about 10 ml. per 15-minute period. It seems that this minimal excretion is not under the control of ADH, and, in order to find that part which is so controlled, this residual excretion must be subtracted from all the observed readings. This has been done in Fig. 3, in which zero time is the time of injection, and the volumes plotted refer to the urinary output in the 15-minute periods, 10 ml. having been subtracted from each

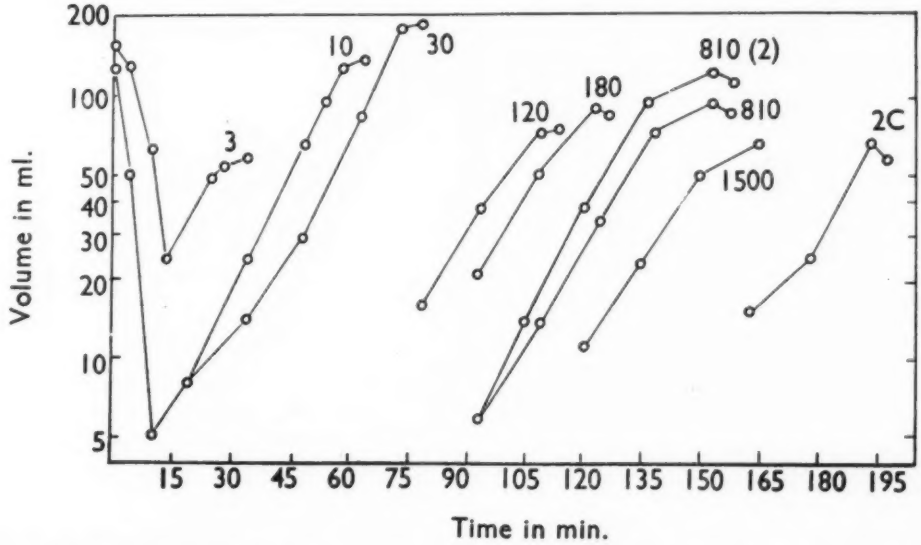


Fig. 3.—Rate of increasing urinary excretion. Ordinates: Urinary output in ml. per 15 min. 10 ml. has been subtracted from the output actually measured, and the remainder has been recorded. Abscissae: Time in min. The figures on the curves relate them to the dose in mU. PLE or to the smoking of two cigarettes.

figure recorded. In some experiments only two points are available, but where more points were obtained the resemblance to an exponential curve is unmistakable. Naturally this rate of increase of urine flow can only be maintained up to the maximum at which the kidney can work; also, it will only continue as long as the subject is hydrated; when the surplus water has been lost, the subject's own pituitary will liberate ADH and stop the diuresis. So the curves are rounded off at the top, and the observed peak of diuresis may occur at any point in its course.

If destruction of ADH proceeds at the same rate as the urinary flow increases, then the fraction of any injected dose that is left, at a time "$t$" is given by the expression

$$y_t = y_o e^{-kt} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where "$y$" is the fraction of the dose at the relevant time ($y_0 = 1$); and $k$ is equal to the slope of the curves in Fig. 3. Actually these slopes are not all the same; $k$ varies from 0.071 for the small doses to 0.048 for the largest dose.

*The rate of inhibition of diuresis.*—It has been noticed for some time that a finite period must elapse before the maximum degree of inhibition is obtained. Indeed, Kelsall (1949) used this phenomenon to compare the effect of injections of PLE with the effect of pain.

The length of the period given by Kelsall was an average figure of 14 minutes with limits of 11 and 23 minutes. The longest period that we observed was 14 minutes for the dose of 3 mU., and always shorter periods for larger doses. A dose of 120 mU., for instance, appears to have an immediate effect. A similar result is described by Verney (1947) in his Croonian lecture. In Fig. 20*b*, page 64 in his paper, there are three assay curves on the dog "Nicky." The time from injection to maximum inhibition is there seen to be 10 minutes for 1 mU., about 8 minutes for 2 mU., and 5–6 minutes for 3 mU.

It would be expected that the blood concentration would rise to a maximum very shortly after the injection of a dose of PLE. The circulation time in man is in the region of 10–30 seconds and mixing of the dose with all the blood should be obtained in about two minutes. Before this time, some blood will contain a higher concentration than the average, so the kidney ought to inhibit diuresis very quickly. A mechanism is therefore needed which will provide a delay of some 10 minutes before maximum inhibition is obtained.

*Theory.*—If we suppose that the ADH which inhibits diuresis is present in the kidney, and not in the blood, then, as the dose of PLE is injected into the blood, it must first be transferred into the kidney.

At a time $t = 0$, a dose $x$ is given. Let $X$ be the quantity remaining in the blood after time $t$, and suppose that in a short time $dt$ a quantity $\alpha X dt$ transferred from the blood to the kidneys.

Then $dX = -\alpha X dt$.

Suppose further that the hormone present in the kidneys is destroyed at a rate proportional to $Z$, the quantity actually present in the kidneys; this rate is $\beta Z dt$ in time $dt$.

Then $dZ = \alpha X dt - \beta Z dt$

Hence $\dfrac{dX}{dt} = -\alpha X$ and $\dfrac{dZ}{dt} = \alpha X - \beta Z$

with conditions that at $t = 0$, $X = x$, $Z = 0$.

The solutions are

$$X = xe^{-\alpha t}$$

$$Z = \frac{\alpha X}{\alpha - \beta}\left(e^{-\beta t} - e^{-\alpha t}\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

It is reasonable to suppose that there is some quantity $Z_0$, such that excretion is inhibited (partially or wholly) as long as $Z > Z_0$. As $t$ increases from zero to moderate values and to infinity, $Z$ increases from zero to a maximum and then decreases again to zero. If the maximum exceeds $Z_0$, there will be inhibition. In general, there will be two times, $t_1$ and $t_2$, at which $Z = Z_0$, and inhibition will continue in the interval between them. Now $t_1$ and $t_2$ are the roots of the equation

$$Z_0 = \frac{\alpha x}{\alpha - \beta}(e^{-\beta t} - e^{-\alpha t})$$

or

$$x = \frac{(\alpha-\beta)Z_0}{\alpha} \cdot \frac{e^{\beta t}}{1-e^{-(\alpha-\beta)t}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

In fact, $t_2$, the time to the peak, is the largest root of this equation. For "large" $x$, $t$ will be large and $e^{-(\alpha-\beta)t}$ small relative to unity. If this term is ignored, then approximately

$$x = \frac{(\alpha-\beta)Z_0}{\alpha}\beta t_2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

The regression equation (1) is presumably an approximation to this. It suggests that $\beta = 0.0545$. Actually $\beta$ might be a little larger to allow for the neglected term, but even if $\beta$ were 0.06 (and $\alpha = 0.2$), $1 - e^{-0.14t}$ would be so near to unity after 50 minutes that it could be safely ignored.

The considerations leading to $\alpha = 0.2$ are as follows: the blood flow through the kidneys is 1.2 l. per minute. As the blood volume is 6 l., a fifth of the blood volume passes through the kidneys per minute. It is supposed that the kidneys extract all the hormone from the blood that passes through them, and that this cleared blood immediately dilutes the remainder. This will not be exactly true in practice, as a finite time must elapse before mixing is obtained and before the cleared portion can again enter the kidney. This will result in an increase of the true value of $\alpha$.

Let $\alpha = 0.2$, $\beta = 0.055$. Then equation (5) also suggests that

$$\frac{0.2-0.055}{0.2} \times Z_0 = 0.285$$

or $Z_0 = 0.4$ mU.

Fig. 4 shows

$$\frac{Z}{x} = \frac{0.2}{0.2-0.055}(e^{-0.055t} - e^{-0.2t}) \quad \text{(from equation 3)}$$

that is, the proportion of the applied dose in the kidneys, undestroyed, at time $t$. The proportion reaches a maximum of about 0.61 at $t = 9$ minutes, and thereafter decays to zero. After $t = 20$, the decay is almost entirely exponential. The smallest dose that produces any inhibition is $x_0$, such that $0.61x_0 = Z_0$, or $x_0 = 0.66$ mU.

It is now possible to see what happens when a small dose is given: 0.66 mU. leads to a maximum value of 0.4 mU. in the kidney in 9 minutes. A slightly smaller dose will not produce enough ADH in the kidney to inhibit diuresis and so will be recorded as having no effect. A slightly larger dose will cause a perceptible effect, and will be recorded as having inhibited diuresis for 9 minutes or more. This is the explanation of the term $e^{-(\alpha-\beta)t}$ in equation (4), for when $t$ is small this expression is large relative to unity and cannot be ignored. It can also be seen that equation (1)

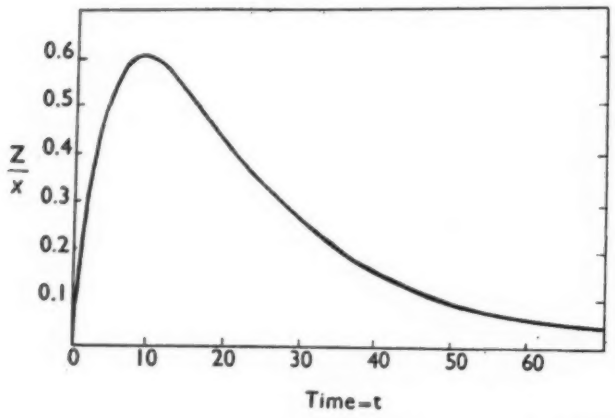


FIG. 4.—Relation between the fraction of an injected dose that is in the kidney and the time that has elapsed since injection. Ordinates: The proportion $Z/x$: Equation (3). Abscissae: Time in min. The fraction reaches a maximum of 0.61 of the injected dose, 9 min. after injection.

is only true when $t$ is large. When $t$ is less than 50 minutes, there ceases to be a linear relation between the time to the peak of diuresis following the inhibition, and the logarithm of the dose injected. Indeed, in reality, there is no linear relation at all; it is only seen because, when $t$ is large, the difference between the theoretical relation and a linear relation is imperceptible.

Some evidence in favour of a non-linear relationship between the time to the peak and the logarithm of the dose, when the dose is small, is given by Verney (1947). Fig. 28 on page 75 of his paper shows some assay curves on the dog "Sally." The times to the peak (which are not, however, very clearly defined) with the doses 0.5, 1.0, and 2.0 mU. are evidently very similar, although the actual curves relating urinary excretion to the time elapsing since the injection are quite separate.

*ADH in the urine.*—It is possible that some ADH is filtered by the glomerulus and washed away in the profuse diuresis which is in progress when the injection is given. This would cause the rate of urine flow to change more slowly at the beginning of the onset of the inhibition than is to be expected from a consideration of the amount of hormone present.

*The delay in the excretion of ingested water.*—The peak of the diuresis which follows the ingestion of water is often hard to define. The peaks recorded in Table II, and to which Fig. 2 and equation (1) refer, related to a rate of urinary flow of about 150 ml. per 15 min. This rate of flow is generally first reached about 45 minutes after the ingestion of water. If at this time 0.4 mU. remains in the kidney ($Z_0$), equation (2) can be used to find out how much ADH was present when the water was ingested. According to the theory, $k$ in equation (2) is equal to $\beta$ in equation (3). As in fact $k$ has a range of values, and the value 0.055 is within this range, no great error is introduced by assuming that $k$ is equal to $\beta$. Then equation (2) shows that 4.8 mU. must have been present, some in the kidney and some still circulating in the blood.

Before the water was ingested, when the amount of hormone in the kidney was presumably constant, the rate of destruction of the hormone equalled the rate at which new hormone was brought by the blood. This gives 3.8 mU. in the kidney and 1.0 mU. in the whole of the blood. The rate of destruction is 0.21 mU. per minute in the kidney, or 3.5 $\mu$U. per second. The order of magnitude of these figures will vary considerably with the degree of hydration in a particular case.

If the time to a urinary flow of 150 ml. per 15 min. was 30 minutes instead of 45, the quantity present would be 2.1 mU., corresponding to a rate of destruction of 1.5 $\mu$U. per second.

*The antidiuretic hormone liberated by nicotine.*—The amount of antidiuretic hormone liberated by nicotine has been calculated from the period of inhibition produced, and in Table IV the results in smoking are tabulated. When the antidiuretic action lasted more than 120 minutes the amount of hormone liberated has been recorded as greater than 1,000 mU. If the amount is determined by extrapolation in Fig. 2 it would be much greater than 1,000 mU., and then would not agree with the amount liberated as calculated from the amount excreted in the urine (see Table IV). It was observed by Burn *et al.* (1945) that the injection of 100 mU. PLE caused an inhibition of 107 minutes, but that when this amount was given in two doses of 50 mU. each, separated by a period of 45 minutes, the period of inhibition was 140 minutes, which is an inhibition shown by the regression line to be caused by a single dose of 600 mU. When nicotine provides a prolonged stimulus to the pituitary as in smoking, then the antidiuretic hormone is likely to be released in a continuous stream throughout the smoking, and when it arrives at the kidney in this way a very much smaller total dose will provide an inhibition of a given duration. Thus, in Exps. 4 and 5, Table IV, the amount of hormone liberated in the blood when calculated from the amount found in the urine was 1,900 mU. If this amount was liberated all at once, it would cause an inhibition of 161 minutes. Actually the inhibition was 195 minutes, which corresponds to the liberation of 11,000 mU. all at once as determined by extrapolation in Fig. 2. If, however, 1,900 mU. were liberated, not all at once but during 23 minutes, it would certainly prolong the inhibition well beyond 161 minutes. Thus the discrepancy between the amount of hormone liberated (1) calculated from the duration of the antidiuretic action, and (2) calculated from the amount excreted in the urine, is probably explained.

## SUMMARY

1. The antidiuretic response of a man to a range of doses of posterior lobe extract given intravenously has been measured in three subjects. A regression line relating dose to antidiuretic effect has been obtained. The action of the antidiuretic hormone has been considered mathematically.

2. The excretion of the antidiuretic hormone in the urine after intravenous injection of posterior lobe extract has been measured. For doses in the range of 180 to 1,500 milliunits the amount excreted was found to vary from 2.6 to 7.4 per cent, the mean figure being 5.0 per cent.

3. Some observations have been made of the amount of antidiuretic hormone excreted in the urine after the subjects had smoked cigarettes, and on other occasions after they had been injected with nicotine. When smoking is carried out so intensely that side-effects such as pallor, sweating, nausea, and giddiness are experienced, the amount of antidiuretic hormone liberated in the blood, as calculated from that which is excreted in the urine, is in the range of 1,000 to 2,000 milliunits. When smoking is carried out ordinarily, the amount liberated by one or two cigarettes, according to the sensitiveness of the subject, varies from 3 to 190 milliunits.

We wish to thank Dr. D. J. Finney for his help with the mathematical treatment in the Discussion.

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# THE EFFECTS OF DITHIOLS ON THE DISTRIBUTION OF MERCURY IN RABBITS

BY

K. R. ADAM*

*From the Department of Pharmacology, University of Edinburgh*

(Received May 16, 1951)

The efficiency of dimercaprol as an antidote in poisoning by mercuric chloride has been established by both experimental (Gilman, Allen, Philips, and St. John, 1946 ; Stocken, 1947 ; etc.) and clinical (Longcope and Luetscher, 1946, 1949) investigations. Gilman *et al.* (1946) also reported that the glucoside of dimercaprol (BAL-Intrav) was more active. The following observations were made in order to discover the fate of mercury in poisoned animals after treatment with these substances. A short report has been published recently by Fitzsimmons and Kozelka (1950) of similar investigations on rats and Rhesus monkeys.

## Materials and Methods

*Materials.*—The dimercaprol was a water-purified sample, and was stored in a refrigerator when not in use. Solutions in 66 per cent (v/v) aqueous propylene glycol were made up freshly as required.

Dimercaprol glucoside was received as the barium salt, contained in nitrogen-filled ampoules. Solutions of the free dithiol were prepared as described by Weatherall and Weatherall (1949). The impurity of the preparations of dimercaprol glucoside necessitated estimations of the thiol content of each solution, by means of iodine titration ; this procedure is probably more satisfactory than calculating the dosage from the weight of barium salt used (Weatherall, 1949).

The radioactive isotope of mercury ($Hg^{203}$) was obtained by neutron irradiation of mercuric chloride at A.E.R.E., Harwell. Radioactive chloride ($Cl^{36}$) was removed by reducing the salt to metallic mercury with stannous chloride. The metal was dissolved in aqua regia, and the solution gently heated almost to dryness. Drying was completed in a vacuum desiccator. The low specific activity (4mC. per g.) of the mercury necessitated the quantitative transfer of the mercuric chloride to the injection solutions, which finally consisted of 1.35 mg. mercuric chloride, incorporating 3–5 $\mu$C. of $Hg^{203}$, per ml., dissolved in 4 per cent (w/v) dextrose. A dose of 1.0 ml. per kg. body weight was injected intravenously over a period of 2–3 minutes.

*Method.*—The treatment of the animals, sampling and ashing of tissues, estimation of radioactivity, and calculation of results followed the procedures reported previously in studies of the distribution of lead (Ginsburg and Weatherall, 1948 ; Adam, Ginsburg, and Weatherall, 1949). The mercury isotope used ($Hg^{203}$) emitted $\beta$-radiation of only moderate energy (0.2 MeV) and counts in the fluid counter were consequently appreciably affected by the densities of the media. A graph was constructed relating the decrease in count to the density ; the density of each sample was measured, using simple

*Present address: Department of Pharmacology, School of Pharmacy, University of London.

hydrometers, and the counts corrected by reference to the graph. On the range of densities encountered, the decreases in count were never greater than 10 per cent.

Preliminary recovery experiments indicated that up to 15 per cent of the mercury could be lost during the ashing process, but that this was reduced to 5 per cent or so by avoiding vigorous boiling. As urine and bile were not ashed, mercury was not lost from these fluids in this way. Duplicate samples of liver and kidney were taken routinely, and from these the standard error of the estimations of mercury was found to be $\pm 14$ per cent. The counting-rate was low, and the time of counting was prolonged only sufficiently to give an error of up to $\pm 10$ per cent. The fractions of the dose accounted for by adding together the calculated amounts in all the organs sampled ranged from 70–90 per cent.

## Results

Results are presented for the distribution and excretion of mercury in 19 rabbits, all of which received a single intravenous dose of 5 $\mu$M (1.0 mg. Hg) mercuric chloride per kg. body weight. This dose usually caused some damage to the kidneys, as shown by the presence of albumin and casts in the urine. No other toxic effects, apart from general apathy, were noted in the poisoned animals. Some of the animals were treated with dimercaprol, and some with dimercaprol glucoside, at different times after the injection of mercuric chloride. Signs of poisoning appeared less definite in the urines of the treated animals, but this impression may be biased.

The results obtained from the control animals are shown in Table I as the concentrations of mercury in $\mu$g. per g. tissue and as the percentage of the dose found in various organs and tissues. Although considerable variation was always found between animals receiving the same treatment, certain general trends can be seen. One hour after the injection of mercury a small quantity of the element remained in the plasma ; but within six hours the amounts had fallen and were generally below the detectable level. Of all the organs and tissues sampled, the kidneys contained by far the highest concentration of mercury, and, after 24 hours, accounted for 20–30 per cent of the mercury absorbed. The liver generally contained about 2 $\mu$g. mercury per g., and this concentration did not alter significantly during 14 days after the injection of mercury. The next highest concentrations usually occurred in the lungs, while heart, spleen, and brain contained only very small quantities. A high concentration of mercury was found in bone marrow after one hour, but this fell to below the detectable level later ; similarly, the concentrations in bone decreased, and only very small amounts of mercury were found in this site after 24 hours. Skin usually held a few per cent of the dose, but skeletal muscle contained only small quantities. The amount of mercury in the gastro-intestinal tract rose during the first few hours as excretion increased, then maintained a fairly steady concentration for a few days. Very small amounts occurred in the stomach, but the stomach contents usually showed a much higher concentration of mercury, perhaps because of the excretion of the metal in the saliva. After 24 hours the small intestine, caecum, and colon contained small and similar concentrations, but again higher concentrations of mercury were generally found in the respective contents. A high concentration was found in the bile after one hour, but thereafter the quantities of mercury in this fluid were below the level of detection.

About 4 per cent of the dose was excreted in the urine within an hour, but this rate slowed considerably, and after 24 hours the urine contained 6–15 per cent

TABLE I

The concentrations and the percentages of the dose of mercury in the tissues of rabbits after the intravenous injection of 5 μM/kg. of mercuric chloride

| Time after injecting mercury: | 1 hr. | | 6 hr. | | 24 hr. | | | | | | 5 days | | 14 days | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rabbit No.: | 367 ♂ | | 371 ♂ | | 361 ♀ | | 365 ♂ | | 369 ♂ | | 363 ♂ | | 359 ♂ | |
| Weight, kg.: | 1.50 | | 1.55 | | 2.55 | | 1.80 | | 1.33 | | 1.87 | | 2.25 | |
| | μg. Hg/g. tissue | % of dose | μg Hg/g. tissue | % of dose | μg.Hg/g. tissue | % of dose | μg.Hg/g. tissue | % of dose | μg.Hg/g. tissue | % of dose | μg.Hg/g. tissue | % of dose | μg.Hg/g. tissue | % of dose |
| Plasma | 0.97 | 3.5 | <0.24 | <1.7 | <0.04 | <0.2 | <0.09 | <0.3 | 0.26 | 1.4 | <0.11 | <0.3 | <0.06 | <0.3 |
| Blood cells | 0.12 | 0.4 | <0.33 | <1.7 | 0.66 | 1.3 | <0.11 | <0.3 | <0.30 | <0.5 | <0.09 | <0.3 | <0.09 | <0.3 |
| Lungs | 0.76 | 0.4 | 1.63 | 0.5 | 0.46 | 0.2 | 0.49 | 0.2 | 2.10 | 1.0 | 1.23 | 0.6 | <0.04 | <0.02 |
| Heart | <0.12 | <0.03 | <0.52 | <0.08 | <0.04 | <0.01 | 0.03 | 0.01 | <0.20 | <0.05 | — | — | — | — |
| Stomach | <0.02 | <0.03 | <0.08 | <0.08 | 0.10 | 0.1 | <0.02 | <0.03 | 0.30 | 0.4 | 0.04 | 0.02 | 0.29 | 0.3 |
| ,, contents | <0.03 | <0.11 | 0.20 | 0.6 | 0.41 | 1.2 | 0.85 | 3.2 | 1.10 | 4.6 | 0.97 | 2.1 | 0.18 | 1.2 |
| Small intestine | 0.08 | 0.2 | 1.18 | 1.9 | 0.29 | 0.6 | 0.51 | 1.0 | 0.61 | 1.6 | 0.40 | 0.1 | 0.35 | 0.4 |
| ,, contents | <0.01 | <0.03 | 1.04 | 0.6 | 0.34 | 0.7 | 0.53 | 0.5 | 1.09 | 0.8 | 0.16 | 0.1 | <0.02 | <0.02 |
| Caecum | — | — | — | — | 0.65 | 0.6 | 0.43 | 0.4 | 0.67 | 0.9 | 0.88 | 0.7 | — | — |
| ,, contents | — | — | — | — | <0.04 | <0.1 | 0.89 | 3.2 | <0.11 | <0.6 | 2.22 | 5.1 | — | — |
| Colon | — | — | 0.29 | 0.1 | 0.20 | 0.1 | 0.36 | 0.2 | 0.66 | 0.4 | <0.09 | <0.02 | <0.03 | <0.02 |
| ,, contents | <0.03 | <0.03 | 0.57 | 0.3 | 1.08 | 0.7 | 1.81 | 0.5 | <0.23 | <0.1 | 1.85 | 0.5 | 0.05 | 0.04 |
| Kidneys | 35.62 | 22.6 | 47.75 | 20.6 | 28.31 | 19.8 | 23.06 | 13.2 | 31.92 | 22.3 | 17.41 | 18.4 | 15.36 | 10.2 |
| Liver | 1.84 | 7.3 | 2.91 | 10.6 | 0.76 | 2.9 | 2.03 | 6.3 | 2.90 | 9.2 | 1.96 | 4.3 | 1.84 | 5.3 |
| Bile | 10.32 | — | <2.01 | — | <0.34 | — | <1.71 | — | — | — | <0.45 | — | <0.81 | — |
| Spleen | <0.80 | <0.03 | <1.22 | <0.08 | 1.35 | 0.04 | <0.65 | <0.03 | <0.16 | <0.05 | <1.01 | <0.06 | <0 33 | <0.02 |
| Skin | 0.92 | 11.1 | 0.32 | 3.9 | 1.25 | 15.1 | 0.10 | 1.2 | 0.51 | 6.2 | 0.24 | 2.9 | 0.22 | 2.6 |
| Brain | <0.05 | <0.03 | — | — | <0.03 | <0.01 | <0.05 | <0.03 | <0.13 | <0.08 | — | — | — | — |
| Bone marrow | 2.58 | 5.2 | <1.18 | <2.3 | <0.24 | <0.5 | <0.36 | <0.7 | <0.51 | <1.0 | <0.45 | <0.9 | <0.13 | <0.3 |
| Skull vault | — | — | — | — | <0.14 | | <0.31 | | <0.28 | | <0.21 | | <0.17 | |
| Epiphyses | 0.50 | 2.9 | 0.42 | 1.5 | 0.11 | 0.2 | <0.05 | <0.5 | <0.09 | <1.1 | <0.05 | 0.7 | <0.03 | <0.2 |
| Diaphyses | 0.45 | | <0.23 | | 0.13 | | <0.09 | | <0.20 | | 0.32 | | <0.05 | |
| Skeletal muscle | <0.04 | <2.0 | 0.13 | 6 8 | <0.03 | <1.7 | 0.12 | 6.6 | <0.07 | <3.9 | <0.04 | <2.4 | <0.03 | <1.9 |
| Excreted, urine | — | 3.9 | — | 7.6 | — | 5.9 | — | 12.4 | — | 15.1 | — | 33.0 | — | 38.6 |
| ,, faeces | — | — | — | — | — | 1.0 | — | 1.1 | — | 0.6 | — | 8.5 | — | 26.7 |
| Injection site | — | 18.8 | — | 26.2 | — | 36.4 | — | 32.4 | — | 16.6 | — | 3.3 | — | 7.8 |
| Total accounted | | 76.3% | | 81.2% | | 85.8% | | 82.4% | | 81.1% | | 80.3% | | 93.1% |

of the dose or 8–18 per cent of the mercury absorbed. Only about two-fifths of the dose was excreted in the urine in 14 days. About 1 per cent of the dose was found in the faeces after 24 hours, but this rate of excretion increased and nearly 27 per cent was excreted in this way in 14 days.

The results obtained from animals treated with dimercaprol are shown in Table II. The doses of dimercaprol, and the times after the injection of mercury at which they were given, are set out in this Table. The concentrations of mercury found in most of the tissues investigated were not significantly different from those occurring in the tissues of the control animals. The concentrations of mercury in the kidneys, however, fell profoundly after treatment. After 24 hours, these organs contained 1–5 per cent of the dose of mercury as compared with 13–22 per cent in the control rabbits. This loss of mercury was paralleled by a greatly increased excretion of mercury in the urine. These actions of dimercaprol were found, though on a smaller scale, when treatment was delayed until nine days after the administration of mercury. The two rabbits killed after five days showed similar amounts of mercury in the urine, but it should be noted that the rabbit (No. 364) treated with dimercaprol retained about 30 per cent of the dose at the site of injection. When dimercaprol was administered three days after the mercury, an increase occurred in the concentration of mercury in the urine, as shown in Fig. 1. Treatment begun nine days after the mercury had a similar effect, but this disappeared rapidly in spite of continued treatment (Fig. 2).

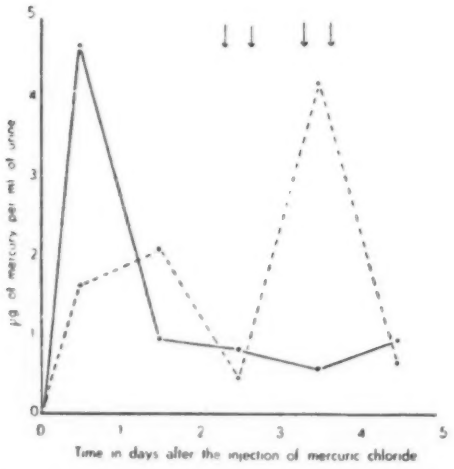


FIG. 1.—The concentrations of mercury found in samples of urine after the intravenous injection of 5 µM/kg. of mercuric chloride. Each arrow represents the injection of propylene glycol or of 25 mg./kg. of dimercaprol. ●———● Rabbit 363 (control). ● - - - - ● Rabbit 364 (treated with dimercaprol).

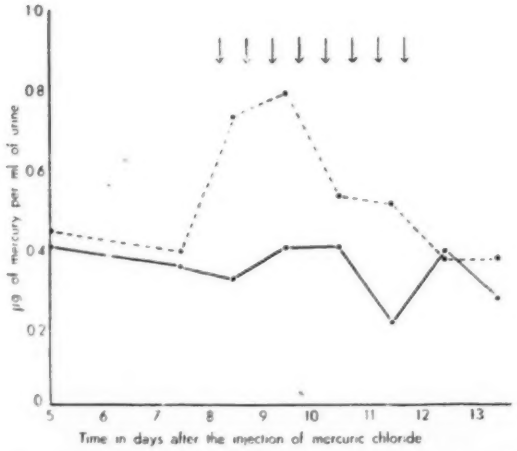


FIG. 2.—The concentrations of mercury found in samples of urine after the intravenous injection of 5 µM/kg. of mercuric chloride. Each arrow represents the injection of propylene glycol or of 12.5 mg./kg. of dimercaprol. ●———● Rabbit 359 (control). ● - - - - ● Rabbit 360 (treated witn dimercaprol).

TABLE II

The concentrations and the percentages of the dose of mercury found in the tissues of rabbits after the intravenous injection of 5 μM/kg. of mercuric chloride, and subsequent treatment with dimercaprol. (50 mg. dimercaprol = 0.4 mM, approximately)

| Time after injecting mercury: | 1 hr. | | 6 hr. | | 24 hr. | | | | 5 days | | 14 days | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment with dimercaprol: | 50 mg./kg. immediately | | 50 mg./kg. at 1 hr., 12.5 mg. kg. at 5 hr. | | 50 mg./kg. at 1 hr., 12.5 mg./kg. at 5 hr. | | 50 mg./kg. at 19 hr., and 12.5 mg./kg. at 23 hr. | | 25 mg./kg. twice daily on days 3 and 4 | | 12.5 mg./kg. twice daily on days 9–12 and once on day 13 | |
| Rabbit No.: Weight, kg.: | 368 ♂ 1.40 | | 372 ♀ 1.86 | | 370 ♀ 2.05 | | 362 ♂ 1.72 | 366 ♂ 2.37 | 364 ♂ 1.78 | | 360 ♂ 1.54 | |
| | μg. Hg/g. tissue | % of dose | μg. Hg/g tissue | % of dose | μg. Hg/g. tissue | % of dose | μg. Hg/g. tissue | % of dose | μg. Hg/g. tissue | % of dose | μg. Hg/g. tissue | % of dose | μg. Hg/g. tissue | % of dose |
| Plasma | 1.66 | 7.1 | <0.19 | <0.9 | <0.10 | <0.5 | <0.07 | <0.3 | <0.13 | <0.5 | <0.08 | <0.3 | <0.06 | <0.3 |
| Blood cells | 2.82 | 7.8 | 1.24 | 2.8 | 2.42 | 5.8 | <0.07 | <0.3 | <0.15 | <0.5 | <0.10 | <0.3 | <0.11 | <0.3 |
| Lungs | 2.41 | 0.9 | <0.18 | <0.07 | <0.08 | <0.03 | <0.04 | <0.02 | 0.53 | 0.2 | <0.06 | <0.02 | <0.05 | <0.02 |
| Heart | 0.34 | 0.07 | <0.27 | <0.07 | <0.14 | <0.03 | <0.06 | <0.02 | <0.08 | <0.02 | — | — | — | — |
| Stomach | 0.71 | 0.8 | 0.20 | 0.2 | <0.03 | <0.03 | 0.16 | 0.2 | <0.03 | <0.02 | <0.03 | <0.02 | <0.06 | <0.06 |
| ,, contents | 0.26 | 1.4 | <0.11 | <0.4 | 0.49 | 2.8 | <0.01 | <0.05 | 0.34 | 0.7 | 0.22 | 0.9 | <0.02 | <0.2 |
| Small intestine | 1.43 | 2.9 | 0.95 | 1.6 | 0.12 | 0.2 | 0.24 | 0.6 | 0.17 | 0.2 | 0.21 | 0.3 | 0.38 | 0.5 |
| ,, ,, contents | 2.65 | 4.1 | <0.12 | <0.07 | 0.96 | 0.5 | 1.02 | 0.8 | <0.04 | <0.02 | 0.13 | 0.2 | 0.21 | 0.3 |
| Caecum | — | — | — | — | <0.36 | <0.4 | <0.01 | <0.02 | <0.03 | <0.02 | 0.01 | 0.01 | — | — |
| ,, contents | — | — | — | — | <0.12 | <0.6 | 0.46 | 2.0 | 0.59 | 1.7 | 0.13 | 0.6 | — | — |
| Colon | 0.64 | 0.3 | 0.58 | 0.3 | <0.07 | <0.03 | <0.02 | <0.02 | <0.06 | <0.02 | <0.07 | <0.02 | <0.03 | <0.02 |
| ,, contents | <0.05 | <0.03 | 0.61 | 0.2 | <0.06 | <0.06 | 0.26 | 0.3 | 1.00 | 0.06 | 0.16 | 0.1 | 0.07 | 0.06 |
| Kidneys | 8.79 | 5.8 | 1.83 | 1.4 | 6.65 | 4.6 | 3.30 | 2.5 | 1.78 | 1.0 | 1.95 | 1.5 | 1.43 | 1.2 |
| Liver | 1.11 | 3.3 | 1.19 | 3.9 | 1.23 | 4.7 | 0.41 | 2.0 | 0.64 | 2.1 | 0.50 | 1.4 | 1.13 | 3.3 |
| Bile | 2.30 | — | <2.22 | — | 1.51 | — | <1.36 | — | <0.85 | — | <1.33 | — | — | — |
| Spleen | <0.81 | <0.03 | <1.64 | <0.07 | 2.99 | 0.3 | <0.64 | <0.04 | <0.83 | <0.02 | <0.40 | <0.06 | <0.42 | <0.03 |
| Skin | 0.52 | 6.3 | <0.29 | <3.4 | <0.15 | <1.8 | <0.04 | <0.5 | 0.33 | 4.0 | <0.09 | <0.11 | 0.05 | 0.7 |
| Brain | <0.07 | <0.03 | — | — | <0.09 | <0.03 | <0.03 | <0.02 | <0.06 | <0.02 | — | — | — | — |
| Bone marrow | <0.30 | <0.6 | <0.63 | <1.2 | <0.45 | <0.9 | <0.35 | <0.7 | <0.32 | <0.6 | <0.32 | <0.6 | <0.20 | <0.4 |
| Skull vault | — | — | — | — | <0.46 | | <0.21 | | <0.27 | | <0.30 | | — | — |
| Epiphyses | <0.05 | <0.4 | <0.18 | <1.2 | <0.09 | <0.9 | 0.03 | 0.05 | <0.04 | 0.8 | <0.06 | <0.6 | <0.05 | <0.3 |
| Diaphyses | <0.09 | | <0.22 | | <0.12 | | <0.06 | | 0.37 | | <0.08 | | <0.06 | |
| Skeletal muscle | 0.32 | 16.7 | 0.50 | 26.3 | <0.07 | <3.9 | 0.08 | 4.4 | 0.11 | 5.6 | 0.07 | 3.9 | 0.18 | 9.4 |
| Excreted, urine | — | 12.8 | — | 34.1 | — | 56.1 | — | 32.1 | — | 33.8 | — | 34.2 | — | 45.7 |
| ,, faeces | — | — | — | — | — | 0.7 | — | 3.7 | — | 0.9 | — | 4.2 | — | 22.9 |
| Injection site | — | 11.7 | — | 19.2 | — | 10.6 | — | 32.4 | — | 36.2 | — | 29.6 | — | 5.6 |
| Total accounted | | 82.0% | | 90.0% | | 86.7% | | 81.0% | | 87.3% | | 76.9% | | 89.7% |

The results of treatment with dimercaprol glucoside are presented in Table III. Considerable differences from the actions of dimercaprol were found. The mercury content of the plasma was consistently raised, and in rabbits, Nos. 400 and 402, the content of the blood cells was also increased. Large increases in the concentration of mercury in the bile occurred, but the amounts of mercury contained in the small intestine, colon, and their contents were generally similar to those found in untreated animals. No definite change in the concentration of mercury in the liver was observed, but the mercury content of the kidneys was diminished to an extent

TABLE III

The concentrations and the percentages of the dose of mercury found in the tissues of rabbits after the intravenous injection of 5 μM/kg. of mercuric chloride, and subsequent treatment with dimercaprol glucoside. (175 mg. dimercaprol glucoside = 0.6 mM, approximately)

| Time after injecting mercury: | 1 hr. | | 24 hr. | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment with dimercaprol glucoside: | 175 mg./kg. immediately | | 175 mg./kg. at 1 hr., and 55 mg./kg. at 5 hr. | | | | 175 mg./kg. at 19 hr., and 55 mg./kg. at 23 hr. | |
| Rabbit No.: | 410 ♂ | | 400 ♀ | | 402 ♀ | | 406 ♀ | |
| Weight, kg.: | 1.85 | | 1.75 | | 1.75 | | 1.75 | |
| | μg. Hg/g. tissue | % of dose | μg. Hg/g. tissue | % of dose | μg. Hg/g. tissue | % of dose | μg. Hg/g. tissue | % of dose |
| Plasma | 3.67 | 12.8 | 1.64 | 6.7 | 1.25 | 4.8 | 2.02 | 8.5 |
| Blood cells | <0.23 | <0.8 | 2.57 | 7.5 | 5.35 | 16.7 | <0.07 | <0.2 |
| Lungs | 1.66 | 0.7 | 0.87 | 0.3 | 1.63 | 0.5 | 0.53 | 0.2 |
| Small intestine | — | — | <0.01 | <0.02 | 1.12 | 1.9 | — | — |
| ,, ,, contents | — | — | 1.10 | 0.5 | 1.25 | 0.4 | — | — |
| Colon | — | — | 0.66 | 0.3 | 1.14 | 0.5 | 0.75 | 0.4 |
| ,, contents | — | — | 1.84 | 1.3 | 7.30 | 1.1 | 0.55 | 0.1 |
| Kidneys | 3.32 | 2.2 | 4.76 | 2.8 | 6.25 | 4.7 | 1.93 | 1.5 |
| Liver | 4.72 | 13.3 | 2.27 | 8.3 | 2.48 | 7.3 | 1.58 | 6.1 |
| Bile | 3.59 | — | 6.29 | — | 2.56 | — | 6.19 | — |
| Skin | 1.42 | 17.0 | 0.66 | 11.4 | 1.80 | 21.6 | 0.47 | 8.8 |
| Skeletal muscle | <0.07 | <3.9 | 0.08 | 4.9 | <0.02 | <1.3 | 0.05 | 3.5 |
| Excreted, urine | — | 17.4 | — | 24.5 | — | 20.2 | — | 29.2 |
| ,, faeces | — | — | — | 2.5 | — | 1.5 | — | 0.2 |
| Injection site | — | 8.1 | — | 0.5 | — | 1.8 | — | 15.3 |
| Total accounted | | 71.5% | | 71.5% | | 83.0% | | 73.8% |

| Time after injecting mercury: | 24 hr. | |
|---|---|---|
| Treatment with dimercaprol glucoside: | 175 mg./kg. at 19 hr., and 55 mg./kg. at 23 hr. | |
| Rabbit No.: | 401 ♀ | |
| Weight, kg.: | 1.72 | |
| | μg. Hg/g. tissue | % of dose |
| Plasma | 1.07 | 4.5 |
| Blood cells | 0.90 | 2.5 |
| Lungs | 1.30 | 0.6 |
| Small intestine | 0.69 | 1.5 |
| ,, ,, contents | 1.37 | 0.4 |
| Colon | 0.80 | 0.4 |
| ,, contents | — | — |
| Kidneys | 4.82 | 3.9 |
| Liver | 1.46 | 5.6 |
| Bile | 14.62 | — |
| Skin | — | — |
| Skeletal muscle | 0.09 | 6.9 |
| Excreted, urine | — | 20.6 |
| ,, faeces | — | 0.9 |
| Injection site | — | 23.8 |
| Total accounted | | 71.6% |

similar to that occurring in dimercaprol-treated animals. The urinary excretion of mercury was increased, though to a smaller extent than by dimercaprol. Two additional rabbits (not shown in the Table) were treated with 175 mg./kg. of the glucoside at one hour, and with 55 mg./kg. at five hours, after the injection of mercury: these animals excreted 32.5 and 42.2 per cent of the dose in the urine in 24 hours. At the same time, another rabbit was treated with 50 mg./kg. of dimercaprol at one hour, and 12.5 mg./kg. at five hours, after the injection of mercury; this animal excreted 56.9 per cent of the dose in its urine during 24 hours. The mean quantities of mercury excreted in the urine in 24 hours, calculated as percentages of the mercury absorbed, were for the control animals 14 per cent, for

the animals treated with dimercaprol 53 per cent, and for those treated with dimercaprol glucoside 30 per cent. All these differences were significant ($P<0.01$).

A summary of the mean percentages of the absorbed mercury found in various tissues after 24 hours, for the three groups of animals, is given in Table IV. The results thus presented tend to be over-simplified, but this may be justified by the more immediate comprehensibility of the data.

TABLE IV

Summary table of the distribution of mercury in rabbits, without and with treatment with dimercaprol or dimercaprol glucoside. The figures given are the mean percentages of the absorbed mercury found in each group of tissues twenty-four hours after the intravenous administration of 5 μM/kg. of mercuric chloride. Details of treatment are as shown in Tables I, II, and III

| Treatment: | None | Dimercaprol | Dimercaprol glucoside |
|---|---|---|---|
| No. of rabbits: | 3 | 3 | 4 |
| Blood | 1.2 | 2.1 | 13.7 |
| Small intestine plus contents, Colon plus contents, Faeces | 4.6 | 3.7 | 4.1 |
| Kidneys | 25.8 | 3.5 | 3.6 |
| Liver | 8.0 | 3.8 | 7.5 |
| Skin, Skeletal muscle | 14.2 | 7.2 | 19.0 |
| Urine | 15.1 | 54.1 | 26.5 |

## Discussion

The literature describing the distribution of mercury in animals is small in quantity and meagre in information. Very small amounts of the soluble inorganic compounds of mercury kill animals within a short period of time, and hence a sensitive analytical method is necessary for such studies.

The results obtained in the present investigations are in accord with those found by Lomholt (1924) after intramuscular injection of various compounds of mercury into rabbits; and with those obtained by Sollmann and Schreiber (1936) by analysis of the tissues of human cadavers after death from mercuric chloride poisoning. They are not consistent with the observations of Young, Taylor, and Merritt (1930) who reported that considerable amounts of mercury were deposited in the bones of rabbits. The paucity of the latter authors' data prevents any attempt to explain this discrepancy.

The striking decreases in the concentrations of mercury in the kidneys which occurred after treatment with dimercaprol have also been reported recently by Fitzsimmons and Kozelka (1950), using rats and Rhesus monkeys. These authors state that the mercury content of other tissues was raised after dimercaprol, but they do not give any details. They also found that rats excreted more mercury in the urine after treatment with dimercaprol, but that the faecal excretion was unchanged. This increased urinary excretion of mercury was found in the present work, and it occurred even when treatment was delayed until nine days after the administration of mercuric chloride. Evidence was also obtained that the increase in the excretion of mercury was transient in spite of continued treatment with dimercaprol.

The decreased amounts of mercury in the kidneys after treatment correspond with the diminished renal injuries noted in rabbits by Ginzler (1946) and by Gilman *et al.* (1946). These authors found that delay in treatment greatly decreased the protection of the kidneys by dimercaprol. It should be noted that the quantities of mercury in the kidneys are still diminished by treatment begun several days after poisoning.

Continued treatment did not maintain a supra-normal excretion of mercury, although a considerable amount of the metal remained in the body. This may be due to part of the mercury being bound more firmly to tissue constituents; or, conceivably, to an increasing ability of the body to detoxicate dimercaprol, but so far no evidence in favour of this is known.

The results reported here support to some extent the hypothesis of an extracellular distribution of dimercaprol glucoside (Danielli, Danielli, Fraser, Mitchell, Owen, and Shaw, 1947). The mercury level in the plasma was consistently raised after treatment with the glucoside, but not after dimercaprol therapy. Considering the size of the dose, and the water-solubility, of dimercaprol glucoside, it is surprising that the excretion of mercury in the urine after treatment with this substance was less than that obtained after dimercaprol therapy. It should be noted that when the glucoside was administered immediately after the mercuric chloride, the quantity of metal in the urine after one hour was similar to that obtained after dimercaprol treatment at a corresponding time. Possibly some type of renal damage occurs within an hour after the administration of mercury, and can be alleviated by treatment with dimercaprol, but not with dimercaprol glucoside. Nevertheless, Gilman *et al.* (1946) found dimercaprol glucoside to be as effective an antidote in mercury poisoning as dimercaprol, in equimolecular doses. As even larger doses of mercury were administered in their experiments, it must be assumed that the mercury retained in the plasma by dimercaprol glucoside is as effectively non-toxic as if it had been excreted in the urine. Possibly this plasma mercury would have been found to diminish, and the metal excreted in the urine increased, had the observations been prolonged beyond 24 hours. In any case, the plasma mercury was maintained at a considerably higher level than that found in the control animals, suggesting either that the dithiol-mercury complex was attached to plasma proteins or that the renal tubules tended to reabsorb it.

## Summary

1. Studies have been made of the distribution and excretion of mercury, using a radioactive isotope, in rabbits, without and with treatment with dimercaprol or dimercaprol glucoside.

2. After the intravenous injection of 5 $\mu$M/kg. of mercuric chloride, the highest concentrations of mercury were found in the kidneys. Of the other tissues sampled, only the liver contained more than 1 $\mu$g. Hg per g. consistently. The excretion of mercury was slow and accounted for only two-thirds of the dose after two weeks, occurring mainly in the urine.

3. After treatment with dimercaprol, much smaller amounts of mercury were found in the kidneys. The excretion of mercury in the urine was greatly enhanced, but the faecal excretion was unaffected. These changes were found even when treatment was delayed until nine days after poisoning.

4. Similar changes occurred after the administration of dimercaprol glucoside, except that the concentrations of mercury in the plasma, and the biliary excretion, increased. The urinary excretion of mercury was less than that obtained after treatment with dimercaprol.

I wish to express my gratitude to Professor J. H. Gaddum and to Dr. M. Weatherall for their advice and criticism ; to the Medical Research Council for a training grant, during the tenure of which this work was carried out ; and to Boots Pure Drug Co. for a gift of dimercaprol glucoside.

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# THE MECHANISM BY WHICH N : N-DIBENZYL-CHLOROETHYLAMINE PROTECTS ANIMALS AGAINST CARDIAC ARRHYTHMIAS INDUCED BY SYMPATHOMIMETIC AMINES IN PRESENCE OF CYCLOPROPANE OR CHLOROFORM

BY

GEORGE FAWAZ

*From the Department of Pharmacology, American University of Beirut, Beirut, Lebanon*

(Received March 30, 1951)

N : N-Dibenzyl-$\beta$-chloroethylamine (" Dibenamine ") protects animals against cardiac arrhythmias produced by cyclopropane-adrenaline (Nickerson and Goodman, 1947). The mechanism of this protection is obscure and will remain so until the nature of these arrhythmias is better understood. Meek (1940–1), Stutzman *et al.* (1947, 1949), and Allen *et al.* (1945) maintain that sympathomimetic amines having a catechol ring produce ventricular tachycardia in a dog under cyclopropane anaesthesia only when the heart is sympathetically innervated. They postulate the necessity of intact reflex pathways for both the dog and the cat since they found that decerebration, cardiac sympathectomy, and partial evisceration are procedures that prevent such arrhythmias. Meek and his collaborators do not consider the pressor effect of adrenaline of great significance in the production of arrhythmias under cyclopropane, for " when (ventricular) tachycardia was prevented by decerebration, ergotamine, or sympathectomy, often both the absolute rise in blood pressure and the angle of steepness of the rise were even greater than when tachycardia had previously been produced " (Meek, 1940–1).

Recently, Moe *et al.* (1948) have revived the idea that the pressor action of adrenaline is an important factor in the production of adrenaline-anaesthetic arrhythmias (see Allen, 1934 ; Shen, 1938 ; Shen and Marri, 1940). They found that if the pressor action of adrenaline was prevented by a pressure stabilizer there was marked—but not complete—protection against idioventricular activity under cyclopropane. Furthermore, they showed that a mechanical elevation of blood pressure after injection of adrenaline produces idioventricular rhythms in spite of pretreatment with an otherwise protective dose of dibenamine. In view of these findings and those of Acheson, Farah, and French (1947) and Nickerson and Goodman (1947) in which it was demonstrated that dibenamine does not inhibit the cardiac chronotropic and inotropic actions of adrenaline, Moe *et al.* concluded that the protective action of dibenamine could be explained on the basis of its ability to inhibit the peripheral, i.e., pressor, action of adrenaline. Furthermore, Moe and Freyburger (1950) found that protection against cyclopropane-adrenaline arrhythmia after upper thoracic sympathectomy is due to a reduction of blood

pressure after the operation and that protection is lost after restoration of blood pressure.

Nickerson and Nomaguchi (1949) conclude that the protective action of dibenamine on cyclopropane-adrenaline arrhythmias is chiefly due to a direct action on the myocardium which is independent of a transient " quinidine-like " activity. The prevention of the pressor response to adrenaline by dibenamine is not the main factor, since small doses of dibenamine can reverse the pressor response to adrenaline without protecting the heart against arrhythmia. Furthermore, N-*iso*propyl*nor*-adrenaline can produce arrhythmias under cyclopropane although it has only a vasodepressor action. On the other hand, Nickerson and Nomaguchi do not deny that the pressor action of adrenaline is a contributing factor in the production of arrhythmia, since larger doses of dibenamine were required to protect—although incompletely—against a standard dose of adrenaline if the blood pressure was raised mechanically by occlusion of the aorta after the injection of adrenaline.

Finally, Murphy, Crumpton, and Meek (1949) conclude that the pressor action of adrenaline " may have a favourable effect on the appearance of (ventricular) tachycardia " ; their experiments utilizing a pressure stabilizer confirm the findings of Moe *et al.* (1948) only in part.

It appeared worth while to try to produce anaesthetic sympathomimetic amine arrhythmias in isolated mammalian hearts (heart-lung preparations) and to discover whether dibenamine exerts a protective action. In such a preparation reflex pathways are absent, and adrenaline causes but a slight rise in blood pressure. Furthermore, dibenamine could cause here no reversal of any pressor action of adrenaline. Moe *et al.* (1948) have produced multifocal ventricular discharges, but no fibrillation, in four dog heart-lung preparations by combining cyclopropane-adrenaline administration with a mechanical rise in blood pressure. They did not study the effect of dibenamine on such arrhythmias.

## METHODS

### *Cyclopropane-adrenaline arrhythmias in heart-lung preparations*

Male and female dogs ranging in weight from 5 to 9 kg. were used. Starling heart-lung preparations were made under sodium pentobarbital anaesthesia by the usual procedure. The temperature of the blood entering the heart was kept between 38 and 38.5° C. The blood volume was about 900 c.c. The resistance was maintained at 6.8 cm. Hg, and the blood pressure ranged between 10 and 12.5 cm. Hg. The venous inflow level was 9 cm. above the opening of the inferior vena cava. At the beginning of the experiment the venous inflow level was raised 10 cm., the output which was usually doubled was measured, and then the level was returned to and kept in the original position for 30 minutes. The level was now raised 10 cm. and kept in this position for the duration of the experiment. Pure $O_2$ was then introduced from a rubber bag to displace the air in the whole system, then a mixture of cyclopropane and $O_2$ was introduced from an 8-litre rubber bag. The concentration of cyclopropane was adjusted so that the system contained $40 \pm 5$ per cent cyclopropane in $O_2$. In order to ensure proper mixing of the gases and adequate oxygenation of the blood the following procedure was employed. The exhaled gas mixture from the pump passed through a soda-lime trap, then through a glass tube 15 mm. in diameter that reached to the bottom of the rubber bag. The inhaled gas passed from the bag into the pump through a second glass tube of the same width. Ten minutes after the introduction of the cyclopropane-oxygen

mixture 0.06 mg. adrenaline in 5 c.c. saline was injected within a period of two seconds into the venous cannula at a distance of 20 cm. from the heart.

When chloroform was used instead of cyclopropane, the method of Melville (1946) for producing ventricular fibrillation in intact dogs was followed. Here the venous inflow level was kept at a height of 9 cm.; 0.02 mg. adrenaline per kg. of the original weight of the dog was injected into the rubber tubing at a point as far from the heart as the usual distance from the point of injection in the femoral vein to the heart in the intact animal.

Electrocardiographic tracings, lead II, were taken in the usual manner.

*Chloroform-sympathomimetic amine arrhythmias in intact dogs*

Here the technique used by Melville (1946) was followed. Artificial respiration was kept at a moderate level so as not to influence the pre-existing blood pressure.

## RESULTS

*Isolated hearts*

*Experiments with cyclopropane.*—Table I shows that arrhythmias can be produced in the isolated dog heart. Fig. 1 illustrates the production of ventricular fibrillation by cyclopropane-adrenaline. The increase in venous pressure and the

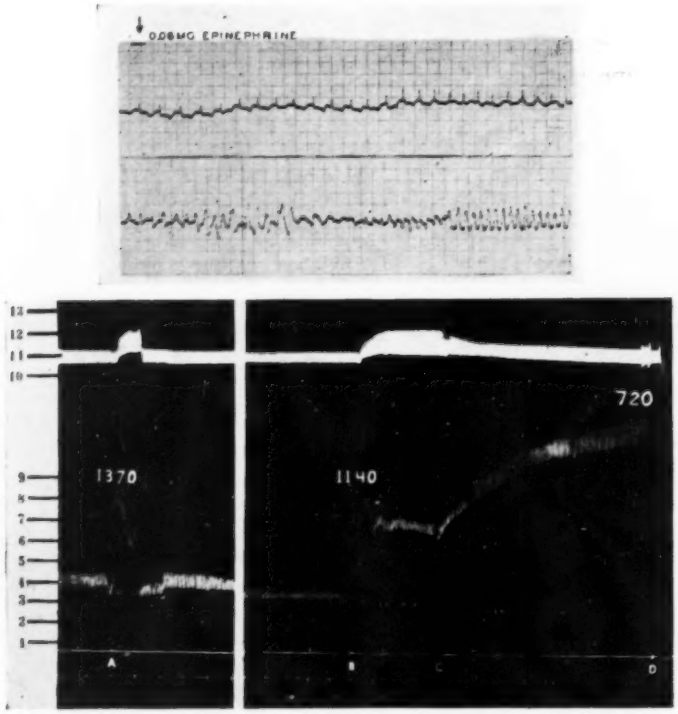


FIG. 1.—Effect of cyclopropane-adrenaline on an isolated heart. Dog 5.2 kg. Heart-lung preparation. *Top:* E.C.G. tracings (lead II) from the time the adrenaline injection was completed. Fibrillation occurred after 15 sec. *Bottom:* Tracings from top to bottom: arterial pressure in cm. Hg; right auricular pressure in cm. $H_2O$; time signal in minutes. At A venous inflow level raised 10 cm.; output at higher level 1,370 c.c. per minute. At B, 30 min. after A, inflow level again raised 10 cm. and kept at high level. At C, cyclopropane-$O_2$ introduced. Output at higher level just before D, 720 c.c. per min. At D, 0.06 mg. adrenaline injected.

simultaneous decrease in output after the introduction of cyclopropane are significant, and are in harmony with the findings of Moe *et al.* (1949). Dibenamine did not prevent the production of arrhythmias under the prevailing experimental conditions, whether it was introduced into the venous reservoir or injected into the animal several hours before the operation.

TABLE I

PRODUCTION OF ARRHYTHMIAS IN HEART-LUNG PREPARATION WITH CYCLOPROPANE-EPINEPHRINE. EFFECT OF DIBENAMINE

F = ventricular fibrillation. v.T. = ventricular tachycardia. Room temperature, 14°–18° C.

| Dog No. | Initial output (with venous inflow level 17 cm.) in c.c./min. | Output after 30 min. in c.c./min. | Output after 10 min. cyclopropane-$O_2$ in c.c./min. | Dibenamine | Arrhythmia | Remarks |
|---|---|---|---|---|---|---|
| 1 | — | 1,100 | 770 | None | F | |
| 2 | 890 | — | — | ,, | F | |
| 3 | — | 1,050 | — | ,, | F | |
| 4 | — | 1,090 | — | ,, | F | |
| 5 | — | 620 | 620 | ,, | F | |
| 6 | 1,510 | 960 | 510 | ,, | F | |
| 7 | 1,240 | 1,080 | 330 | ,, | v.T. | |
| 8 | 1,300 | 1,230 | 1,050 | ,, | F | |
| 9 | 1,270 | 1,270 | 1,060 | ,, | F | |
| 10 | 1,580 | 1,300 | 1,080 | ,, | v.T. | |
| 1 | — | 940 | — | 45 mg. | v.T. | Dibenamine added to venous reservoir during 30 min. |
| 2 | 1,320 | 1,200 | 456 | 35 ,, | F | |
| 3 | 1,340 | 1,190 | 1,020 | 20 mg./kg. | F | |
| 4 | 1,270 | 1,170 | 1,030 | 30 ,, | F | |
| 5 | 624 | 624 | 200 | 30 ,, | v.T. | Dibenamine injected 5–17 hr. before operation |
| 6 | 1,380 | 1,180 | 1,170 | 30 ,, | F | |
| 7 | 900 | 840 | 320 | 30 ,, | F | |
| 8 | 1,010 | 900 | 460 | 30 ,, | v.T. | |
| 9 | 1,560 | 1,250 | 840 | 30 ,, | F | Both donor and "heart-lung dogs" injected with dibenamine |
| 10 | 1,390 | 1,200 | — | 30 ,, | F | |

In three control experiments performed during the same period, heart-lung preparations were equilibrated with a mixture of 40 per cent $N_2$ and 60 per cent $O_2$. Injection of adrenaline produced only sinus tachycardia.

The experiments reported in Table I were performed in an unheated laboratory in the winter of 1949–50. Those in Table II were performed in July, 1950. It can be seen that at a room temperature of 29–30° the severity of the arrhythmias was not as great as in winter. Eight experiments performed in the cold room gave evidence of this effect of temperature, the nature of which is not clear. Nickerson and Smith (1949) reported a "seasonal variation in the severity of cyclopropane-epinephrine arrhythmias." Other investigators have also stressed the importance of temperature in influencing ventricular conduction (de Boer, 1923).

*Experiments with chloroform.*—It is also possible to produce arrhythmias in heart-lung preparations with chloroform and adrenaline (0.02 mg./kg.). The

TABLE II

EFFECT OF TEMPERATURE ON THE PRODUCTION OF ARRHYTHMIAS IN HEART-LUNG PREPARATIONS BY CYCLOPROPANE-EPINEPHRINE

F = ventricular fibrillation. v.T. = ventricular tachycardia. v.P.S. = ventricular premature systole. v.Fl. = ventricular flutter

| Dog No. | Initial output (with venous inflow level 17 cm.) in c.c./min. | Output after 30 min. in c.c./min. | Output after 10 min. cyclopropane-$O_2$ in c.c./min. | Arrhythmia | Room temp. °C. |
|---|---|---|---|---|---|
| 1 | 1,340 | 1,320 | 1,030 | v.T. | 29–30 |
| 2 | 1,200 | 960 | 540 | Few v.P.S. | 29–30 |
| 3 | 1,100 | 1,080 | 960 | Few v.P.S. | 29–30 |
| 4 | 1,080 | 960 | 720 | Many v.P.S. | 29–30 |
| 5 | 980 | 820 | 480 | F | 29–30 |
| 6 | 1,400 | 1,320 | 820 | Few v.P.S. | 29–30 |
| 7 | 1,080 | 1,100 | 180 | F | 29–30 |
| 8 | 810 | 910 | 310 | Few v.P.S. | 29–30 |
| 9 | 840 | 800 | 620 | Many v.P.S. | 24 |
| 10 | 1,370 | 1,140 | 720 | F | 24 |
| 1 | 1,200 | 1,080 | 170 | F | 14–16 |
| 2 | 1,370 | 1,180 | 540 | F | 14–16 |
| 3 | 780 | 1,066 | 744 | v.T. | 14–16 |
| 4 | 1,340 | 1,320 | 720 | v.T. | 14–16 |
| 5 | 960 | — | 670 | v.T. | 14–16 |
| 6 | 912 | 1,200 | 840 | F | 14–16 |
| 7 | 600 | 480 | 216 | v.Fl. | 14–16 |
| 8 | 1,152 | 1,056 | 840 | F | 14–16 |

arrhythmias noted were premature systoles (4 out of 10 preparations) and ventricular fibrillation (5 out of 10 preparations). However, no sustained ventricular tachycardia was obtained. The same was true of experiments with intact animals to be described below. Here again in five control experiments where adrenaline was injected without chloroform, only sinus tachycardia was produced.

N-*Iso*propyl*nor*adrenaline (isoprenaline), a substance causing vasodepression in intact animals but producing positive chronotropic and inotropic actions on the isolated heart (Konzett, 1940), also caused ventricular fibrillation in three out of six isolated hearts.

Ephedrine in a dose of 2 mg./kg. produced no arrhythmias, not even a single ventricular premature systole in all six isolated hearts. This finding is in harmony with those of Meek (1940–1) on intact animals using cyclopropane.

*Intact animals*

The infusion of dibenamine at the rate of 1 mg./kg./min. five hours before the experiment (total dose, 30 mg./kg.) protected dogs against arrhythmias produced by chloroform-adrenaline in four out of five animals. The blood pressure before adrenaline administration was low as compared with the controls. Adrenaline here caused no rise in blood pressure.

*l-Nor*adrenaline (0.02 mg./kg.) can also produce severe arrhythmias, like those obtained with adrenaline, under comparable conditions. Dibenamine here affords

protection (7 out of 10 dogs) although it only diminishes the pressor action of *nor*adrenaline and does not reverse it. Here again the effect of dibenamine in reducing the blood pressure of dogs, before the injection of *nor*adrenaline, was clearly seen.

A slightly higher dose of *nor*adrenaline (0.03 mg./kg.) produced ventricular fibrillation in 11 out of 13 dogs and many ventricular premature systoles in the other two. When dibenamine (20 mg./kg.) was infused at the rate of 1 mg./kg./min. 30 minutes before the injection of *nor*adrenaline (0.03 mg./kg.) ventricular fibrillation occurred in only two out of twelve dogs ; no arrhythmia was observed in six dogs, and in only one of the remaining four were frequent ventricular premature systoles obtained. The average blood pressure of dogs which had received dibenamine (92 mm.) was less than that of the controls (130 mm.), and the rise in blood pressure produced by *nor*adrenaline was also less (40 mm. against 63 mm. in the controls).

Isoprenaline in combination with chloroform produced no arrhythmias whatsoever in a series of 12 experiments. The doses ranged from 0:02 to 0.07 mg./kg. The blood pressure fall was marked in all experiments, reaching as low a level as 25 mm. Hg in some.

## Discussion

There is no reason to believe that the arrhythmias produced in isolated hearts as reported in this work or those reported by Moe *et al.* (1948) are qualitatively different from those produced in the intact animals. In the first place, adrenaline alone produces only a sinus tachycardia. For the production of arrhythmias, cyclopropane or chloroform are required. In the second place, adrenaline and isoprenaline, but not ephedrine, can produce arrhythmias, a finding in harmony with the results obtained by Meek (1941), who found that only sympathomimetic amines containing a catechol nucleus produced arrhythmias in intact animals. Furthermore, the dosage of adrenaline chosen, which was equivalent to that used for an intact animal, was not excessive, since in our experiments ventricular fibrillation, when it occurred, did so less than 30 seconds after the injection of adrenaline. Since in our system it takes about one minute for the blood to make one complete circuit, the adrenaline must have produced the arrhythmia by one passage through the heart. This finding is in agreement with the interpretation of Nickerson and Nomaguchi (1949). It may perhaps be appropriate to say that it is more difficult to elicit arrhythmias in an isolated heart than in an intact animal.

Nickerson (1949) maintains that dibenamine exerts a direct protective action on the myocardium. He states further that this effect appears to be correlated with its adrenergic blocking action since it is irreversible and non-competitive. Now, if dibenamine acted on the myocardium directly and irreversibly it should have a protective action on the isolated heart whether it was added directly to the venous reservoir or administered several hours before the operation. Furthermore, Acheson, Farah, and French (1949) found that none of the known cardiac actions of adrenaline (viz., positive chronotropic, inotropic, bathmotropic, and dromotropic actions) is influenced by dibenamine.

In view of all this, it is difficult to attribute the protective action of dibenamine in the intact animal to a direct effect on the myocardium. The peripheral actions of dibenamine are probably responsible for its protective action.

In the experiments with chloroform, isoprenaline, which is a vasodepressor, produced no arrhythmias in the intact animal. In the heart-lung preparation, however, where isoprenaline exerts no depressor activity but positive chronotropic and inotropic actions, arrhythmias were produced. On the other hand, dibenamine protected the animal against *nor*adrenaline-chloroform arrhythmia although it only diminished the pressor action of *l-nor*adrenaline.

## Summary

1. Arrhythmias can be produced in dog heart-lung preparations by certain sympathomimetic amines when cyclopropane or chloroform is used as anaesthetic.

2. N : N-dibenzyl-$\beta$-chloroethylamine (Dibenamine) protects the intact animal against cyclopropane-adrenaline arrhythmias, but it does not protect the isolated heart.

3. N-*Iso*propyl*nor*adrenaline (isoprenaline) does not produce arrhythmias in the intact dog under chloroform, but it does so in the isolated heart.

4. Dibenamine protects the intact dog against *l-nor*adrenaline-chloroform arrhythmias, although it only diminishes the pressor action of *nor*adrenaline and does not reverse it.

5. Forty per cent cyclopropane in oxygen decreases the work capacity of the isolated dog heart.

I am indebted to Smith, Kline, and French Laboratories, Philadelphia, Pa., for a generous supply of Dibenamine and to the Sterling-Winthrop Research Institute, Rensselaer, N.Y., for the *l-nor*adrenaline. Grateful acknowledgment is made to Mr. L. Artinian for valuable assistance.

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# COMPOUND 48/80: A POTENT HISTAMINE LIBERATOR

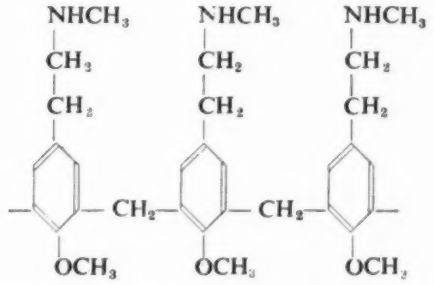
BY

W. D. M. PATON

*From the National Institute for Medical Research, Mill Hill, London, N.W.7*

(Received May 18, 1951)

Baltzly, Buck, de Beer, and Webb (1949), analysing the depressor action of certain *iso*quinoline derivatives, found that this action was due to contamination with phenylethylamine condensation products. Several such compounds were prepared, and one of them (Compound 48/80) was found to lower the dog's blood-pressure in very small doses. Compound 48/80 was obtained from the condensation of *p*-methoxyphenethyl-methylamine with formaldehyde, and has a structure of the general type:



Compound 48/80 is probably a mixture of the dimer, trimer, and tetramer.

The present experiments deal with a pharmacological examination of this substance, and have shown that it owes its depressor activity to its ability to release histamine, and that it is the most active histamine-liberator so far investigated. Since then, Feldberg and Paton (1951) have used this substance to demonstrate the release of histamine from the cat's isolated perfused skin preparation.

## METHODS

These were as described by MacIntosh and Paton (1949), and Feldberg and Paton (1951).

## RESULTS

### *Experiments on the cat*

*Effects on the blood-pressure.*—The injection of 0.1 mg. Compound 48/80 intravenously into a cat anaesthetized with chloralose caused a profound and rapid fall in blood-pressure, beginning 15–20 seconds after the injection. This delayed depressor response was always seen with an effective dose of the drug, whether the dose was big or small. It was usually followed immediately by partial recovery of

the blood-pressure ; but complete recovery was always slow except with very small doses.

A characteristic delayed fall in blood-pressure of 30 mm. mercury could be produced with as small a dose as 9 μg./kg. intravenously (Fig. 1). When the same dose was repeated three minutes later, a delayed depressor response was obtained again, but it was greater in magnitude and longer-lasting than after the first dose.

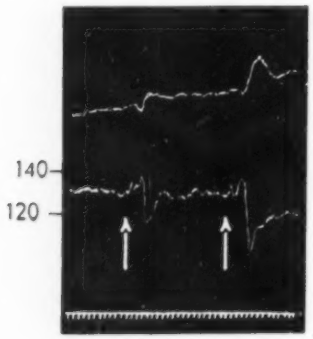



FIG. 1.—Cat, chloralose. Limb volume (upper record) and arterial blood-pressure. At arrows, 9 μg./kg. of Compound 48/80. Time 10 sec.

The depressor response was evidently due to a peripheral vasodilatation, for during the fall in blood-pressure the volume of a limb of the cat increased, waning again as the blood-pressure recovered. Fig. 1 illustrates this point for two small doses of Compound 48/80. Larger doses produced larger and more sustained effects. The heart rate was not altered until the blood-pressure had fallen considerably, when cardioacceleration usually occurred. Even with doses causing a prolonged fall in blood-pressure, the heartbeat remained vigorous.

After a dose of 50 μg./kg., which caused a long-lasting fall in blood-pressure, it was observed that the pressor action of adrenaline was reduced. Thus in one experiment, before the injection of Compound 48/80, 5 μg. adrenaline raised the pressure by 70 mm. from 125 mm. ; after the injection of 0.1 mg. Compound 48/80 the blood-pressure fell to 60 mm., but despite this reduction in blood-pressure, 5 μg. adrenaline now only caused a rise of 30 mm. ; not until 40 minutes later, when the blood-pressure had recovered to 85 mm., did the effect of this dose of adrenaline return to normal. After the injection of histamine liberators in general, and Compound 48/80 in particular, there is almost always a vigorous cardioacceleration during the later part of depressor effect, and it is highly probable that this is due partly to a release of adrenaline (or *nor*adrenaline) in response to the fall of blood pressure and to circulating histamine. Since this reaction must contribute substantially to the subsequent recovery of the blood-pressure, it is possible that the depression of the effects of such released adrenaline by Compound 48/80 contributes to its long duration of action compared to other histamine liberators.

*Haematocrit.*—After large doses of Compound 48/80, the proportion of red cells to plasma in the blood rose. For instance, in Exp. 2 of Table I, the haematocrit increased from an initial value of 44 per cent before the injection to a value of 51 per cent one minute after the intravenous injection of 0.7 mg./kg. of Compound 48/80. In five minutes it rose further to 58 per cent and was at the same value 24 minutes later.

*Release of histamine in the cat.*—The delayed depressor response, the "self-potentiation," the peripheral vasodilatation and the rise in haematocrit are characteristic of histamine liberators (MacIntosh and Paton, 1949). Experiments were therefore made to test whether histamine could be demonstrated in the plasma of animals receiving the drug. Table I gives the results from two such experiments. The plasma before the injection of Compound 48/80 had a histamine equivalent of 0.05 μg./c.c., or less. After the injection of 0.5 or 0.7 mg./kg. of Compound 48/80

TABLE I

| Exp. | Dose of 48/80 | Assay method | Histamine equivalents (μg./c.c.) of plasma before and after injection of 48/80 | | | | |
|---|---|---|---|---|---|---|---|
| | | | Time after injection of 48/80 (minutes) | | | | |
| | | | Before | 1 | 5 | 10 | 30 |
| 1 | 0.5 mg./kg. | Guinea-pig's ileum | 0.05 | 0.5 | — | 0.32 | 0.22 |
| 2 | 0.7 mg./kg. | Guinea-pig's ileum | <0.05 | 0.9 | ≏0.5 | — | <0.1 |
| | | Cat's B.P. | <0.1 | 1.0 | 0.3 | — | <0.1 |

intravenously, it rose within a minute to 0.5 μg./c.c. and 1.0 μg/c.c. respectively in the two tests. That the active substance in the plasma was in fact histamine was shown by the facts that assays on cat's blood-pressure and on guinea-pig's ileum agreed quantitatively, and that small doses of mepyramine abolished its effect on guinea-pig ileum (Fig. 2*b* and *g*) and on the cat's blood-pressure (Fig. 3*b* and *h*) proportionately with the effect of an equipotent dose of histamine. Atropine did not reduce the effect of the plasma on the gut.

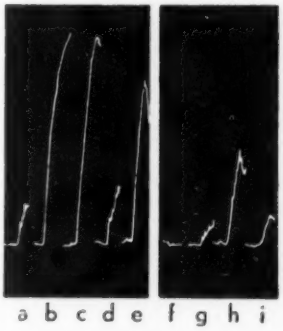



FIG. 2.—Guinea-pig's ileum. Effects of plasma from Exp. 2, Table I. (*a*), (*b*), (*c*), (*d*), 0.1 c.c. plasma withdrawn respectively before and 1 min., 5 min., and 30 min. after the intravenous injection of Compound 48/80. (*e*) 0.06 μg. histamine. Mepyramine 0.3 μg. added to bath thereafter. (*f*) 0.06 μg. histamine. (*g*), (*h*), (*i*), 0.1 c.c. of 1 min., 5 min., and 30 min. plasma respectively.

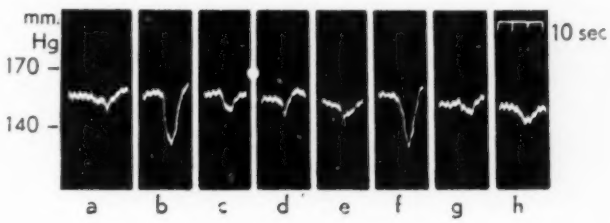



FIG. 3.—Cat, chloralose. Arterial blood-pressure. Intravenous injections. Effects of plasma from Exp. 2, Table I. (*a*), (*b*), (*c*), (*d*), 0.25 c.c. plasma withdrawn before and 1 min., 5 min., and 30 min. after intravenous injection of Compound 48/80. (*e*) 0.05 μg. histamine. (*f*) 0.3 μg. histamine. Between (*f*) and (*g*), 0.05 mg. mepyramine. (*g*) 0.3 μg. histamine. (*h*) 0.25 c.c. of 1 min. plasma.

With samples withdrawn more than one or two minutes after the injection, the determination of the histamine equivalent of the plasma was not so simple. In both experiments of Table I a substance appeared in the later samples of plasma which caused a contraction of the guinea-pig's ileum even in the presence of mepyramine. This substance was present in such large amounts in Exp. 2 of Table I as to be responsible for almost half the activity of the five minutes plasma on the gut (Fig. 2*c* and *h*). The assay on the guinea-pig's gut of the histamine equivalents of these later samples was therefore approximate, and took into account how far the response of the gut to the plasma sample was maintained after mepyramine.

The contraction produced by this unknown substance was much slower than that produced by histamine, and diminished in size rather rapidly if it was elicited at intervals of less than five minutes. This "slow-contracting substance" did not, however, depress the cat's blood-pressure relative to histamine so strongly as it affected the gut, as can be seen by comparing the effects of the one minute and five minutes sample of Exp. 2 on the gut (Fig. 2*b* and *c*) with their effects on the blood-pressure (Fig. 3*b* and *c*). In Exp. 1, this additional substance was present in considerable amounts in the plasma taken both 10 and 30 minutes after the injection of Compound 48/80; in Exp. 2, more activity was present after five minutes than at 1 minute or 30 minutes. It is possible that the persistent depressor effect of Compound 48/80 is partly due to the presence of this substance in the plasma, in addition to circulating histamine.

*Experiments on the dog*

In experiments with other histamine liberators it had been found that when these compounds are injected intravenously in the dog they produce a rise in portal pressure and a delay in coagulation of the blood, as well as causing a fall in blood-pressure and the appearance of histamine in the plasma. Fig. 4*a* shows that the depressor action of Compound 48/80 is likewise associated with a rise in intraportal pressure. When the same dose was repeated 60 minutes later, the animal no longer responded to it, and there was neither a fall in blood-pressure nor a rise in intraportal pressure (Fig. 4*b*).

At the same time as the depressor response to the first dose of Compound 48/80, histamine appeared in the plasma of blood withdrawn from a femoral artery. Fig. 5 shows part of the assay of this plasma on the guinea-pig's ileum, and Fig. 6 shows the results graphically. One minute after the injection, the histamine equivalent of the plasma reached its maximum of 2.2 $\mu$g./c.c., and fell thereafter fairly rapidly. At the same time, the coagulation time lengthened; but whereas the histamine reached its highest concentration in the plasma very rapidly, the prolongation of clotting time was greatest in the sample eight minutes after the injection, and then fell more slowly. One hour later, when the dose of Compound 48/80 was repeated, and failed (as noted above) to have any vascular effect, there was also no significant change in plasma histamine or clotting time.

As in the experiments with cats, there also appeared in the plasma samples a gut-contracting substance, causing a slower contraction than that due to histamine, often diphasic, which was resistant to mepyramine. It was not present, however, in great amounts, and appeared in appreciable quantities only in the sample taken 40 minutes after the first injection. It was not possible to compare accurately the

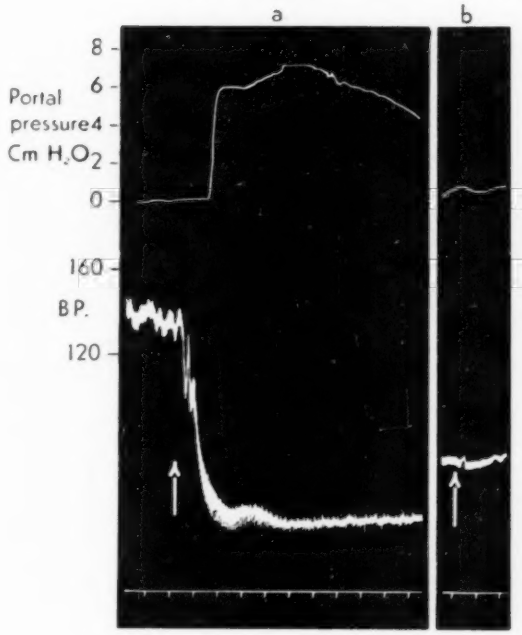



FIG. 4.—Dog, chloralose. Portal pressure (above), arterial blood-pressure (below). (*a*) At arrow, 1.0 mg./kg. Compound 48/80. (*b*) 60 min. later, the same. Time, 30 sec.

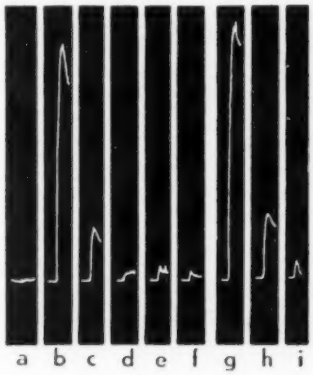



FIG. 5.—Guinea-pig's ileum. Effects of plasma from experiment of Fig. 4. (*a*), (*b*), (*c*), (*d*), (*e*), 0.1 c.c. plasma withdrawn before and 1 min., 7 min., 20 min., and 40 min. after intravenous injection of Compound 48/80. (*f*) 0.1 c.c. plasma withdrawn 2 min. after second injection of Compound 48/80 (*g*), (*h*), (*i*), 0.25 μg., 0.05 μg., 0.01 μg. histamine respectively.

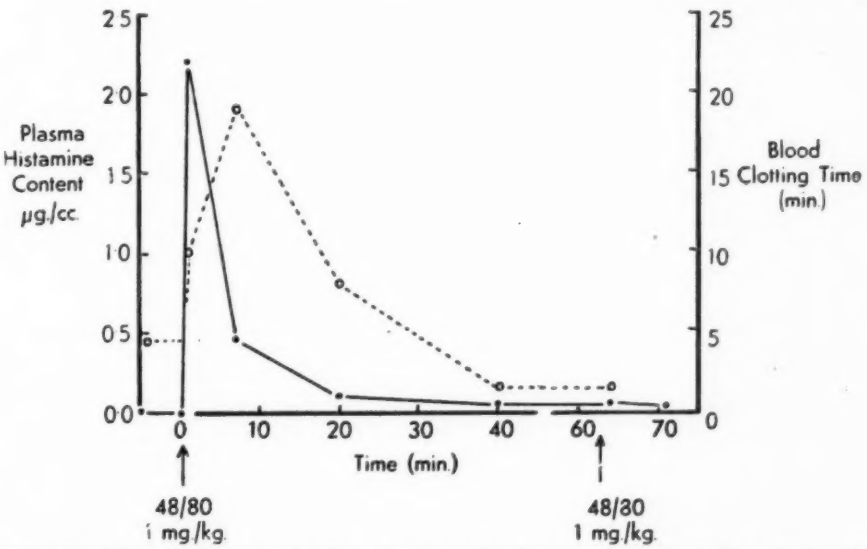



FIG. 6.—Graph of histamine concentration in plasma (μg./c.c. continuous line) and blood clotting time (minutes, dotted line) after injection of 1.0 mg./kg. of 48/80 intravenously at arrows; same experiment as Fig. 4.

amounts of this " slow-contracting substance " in different samples, for it was found that Compound 48/80 in quite small doses itself antagonized the effects of this substance. In Fig. 7 may be seen how 10 $\mu$g. Compound 48/80 reduced by two-thirds the slow contraction produced by the 40 minutes plasma in the presence of mepyramine.

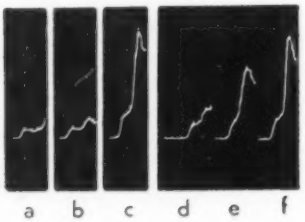



FIG. 7.—Guinea-pig's ileum, in presence of $2.5 \times 10^{-8}$ mepyramine. 0.5 c.c. plasma from experiment of Fig. 4, withdrawn (*a*) before, (*b*) 1 min. after, (*c–f*) 45 min. after injection of Compound 48/80. Between (*c*) and (*d*) 10 $\mu$g. 48/80 added to the bath. 48/80 removed from bath between (*d*) and (*e*).

*Release of histamine from skin*

Compound 48/80, in a dose of 10 $\mu$g., injected intra-arterially into the perfused skin preparation of the cat caused the appearance of 160 $\mu$g. histamine in the venous effluent (Feldberg and Paton, 1951). This experiment was repeated on another preparation, and 65 $\mu$g. histamine was released from the skin, representing 52 per cent of the total histamine present in the portion of skin used. Detectable histamine release can be obtained with even smaller doses of Compound 48/80 ; in a third experiment 1 $\mu$g. Compound 48/80 was injected, and 7.7 $\mu$g. histamine base was collected in the venous effluent.

Compound 48/80 injected intradermally in low concentrations into human skin produces a weal and flare of a kind resembling that produced by histamine. Thus, injected intradermally into the skin of the forearm of two subjects, Compound 48/80 in a dilution of 1 : 1,000,000 caused weals of 7–9 mm. diameter surrounded by flare of 30 mm. diameter ; as the weal developed there was a distinct itchiness at the site of injection. These effects were comparable to those produced by histamine 1 : 1,000,000 or 1 : 300,000.

*Experiments on the isolated guinea-pig's ileum*

Compound 48/80 does not itself cause any contraction of the guinea-pig's ileum, even in a dose of 1 mg. added to a 15 c.c. bath (Fig. 8*f*). But it does possess some power of antagonizing the action of acetylcholine on this preparation. Fig. 8 shows the reduction by 50 $\mu$g. Compound 48/80 of the contraction of the gut

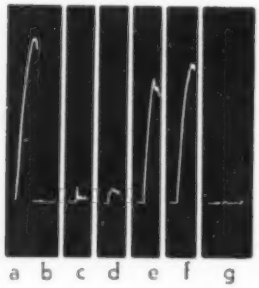



FIG. 8.—Guinea-pig's ileum in 15 c.c. bath. (*a*) and (*c–f*), 0.1 $\mu$g. acetylcholine; (*b*) 50 $\mu$g. and (*g*) 1 mg. Compound 48/80.

produced by 0.15 μg. acetylcholine; 10 μg. Compound 48/80 had a smaller but still distinct effect of the same sort; the $pA_2$ for this atropine-like action, after a two minute exposure, was estimated to be approximately 6. The effect of the drug was sometimes greatest just after it had been washed out, rather than when the response to acetylcholine was tested in its presence, and later there was also observed, during the recovery of the gut, a potentiation of the response to acetylcholine before it finally returned to normal. The response to histamine was also depressed by 50 μg. Compound 48/80, but to a smaller extent; 20 μg. had no detectable effect (Fig. 9).

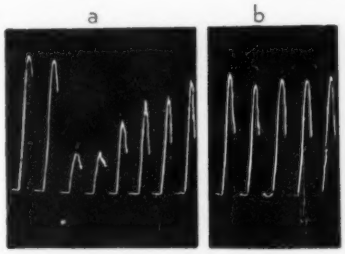



FIG. 9.—Guinea-pig's ileum in 15 c.c. bath. Contractions due to 0.1 μg. histamine. (*a*) 50 μg. Compound 48/80 added before 3rd and 4th contraction. (*b*) 20 μg. Compound 48/80 added before 3rd and 4th contraction.

A conspicuous activity of this drug on the small intestine was that of antagonizing the effects of "slow contracting substances." Fig. 7 showed the reduction by 10 μg. Compound 48/80 of the slow contraction of the gut produced in the presence of mepyramine by plasma from a dog shocked with Compound 48/80. Similar antagonisms are shown in Fig. 10, using doses of 10–100 μg. Compound 48/80 on the slow contractions produced by (*a*) substance P; (*b*) and (*c*) the "slow contracting substances" in extracts of muscularis mucosae of the dog's small intestine (this

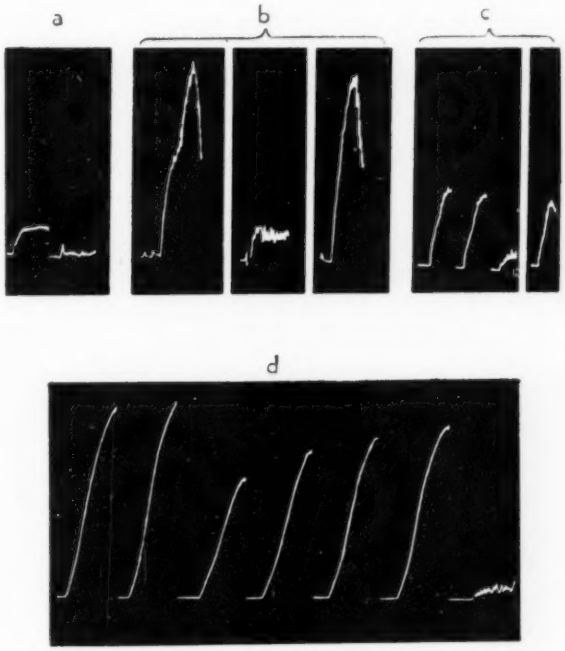



FIG. 10.—Guinea-pig's ileum in 15 c.c. bath. (*a*) Mepyramine $1.5 \times 10^{-9}$. Contractions produced by 1 mg. substance P. Before second contraction, 50 μg. Compound 48/80. (*b*) Atropine $2 \times 10^{-9}$: mepyramine $2 \times 10^{-9}$. First and second contractions produced by extract from 10 mg. muscularis mucosae of dog's small intestine, third contraction by extract from 20 mg. Before second and third contraction, 20 μg. Compound 48/80. (*c*) Atropine $1.5 \times 10^{-9}$: mepyramine $1.5 \times 10^{-9}$. Contractions produced by extract from 50 mg. of whole wall of cat's jejunum. Before third contraction, 30 μg. Compound 48/80. (*d*) Contractions produced by 200 μg. Bradykinin; before third contraction, 10 μg. Compound 48/80; before seventh contraction 100 μg. Compound 48/80.

is probably substance P ; cf. Douglas, Feldberg, Paton, and Schachter, 1951), and in an extract of the whole wall of a cat's jejunum ; (*d*) bradykinin. An extended comparison of the potency of Compound 48/80 against these various contractions was not made. But Compound 48/80 appeared to reduce all these slow contractions by about the same amount, and this antagonism was about as effective as that against acetylcholine and slightly more effective than that against histamine.

## Discussion

The principal action of Compound 48/80 is that of histamine release, verified directly by plasma assay. To this are due all its vascular effects, viz., the delayed depressor response, limb vasodilatation, rise in haematocrit in the cat, and rise of portal pressure in the dog. When, as in the dog which has already received a dose of the drug (Fig. 4), no histamine release into the blood takes place, there is also no depressor action by the drug. Like the other histamine liberators already described, it also causes in the dog a delay in the clotting of blood. In all these respects Compound 48/80 resembles the other members of the group of histamine liberators. Its main interest lies in its remarkably high activity in causing the release of histamine, for this is high by any pharmacological standard. But it also possesses another property, that of causing the appearance of a slow-contracting substance in the plasma of cat and dog. A similar substance has been shown by Beraldo (1950) to appear in the plasma of dogs exposed to anaphylactic shock or injected with peptone. MacIntosh and Paton (1949), reviewing the wide range of bases for which they had obtained evidence of histamine liberation, concluded that there was probably considerable significance to be attached to the existence of so many substances capable of reproducing the salient features of anaphylactic shock. This conclusion is now further strengthened by the observations that histamine liberators may exist, not only of great diversity, but also of great potency, and that the processes of anaphylaxis and of histamine-liberation have yet another characteristic in common, that of causing the appearance in the plasma of a "slow-contracting substance."

The high potency of Compound 48/80 may have some relevance to the mechanism of histamine release. One of the possible ways in which this might occur is by competition of the histamine liberator for the sites in the tissue with which histamine is usually associated. On such a view it is hard to see how a histamine liberator could release, for each molecule injected, more than one molecule of histamine. Indeed, a lower rate of release would be expected, since it could only be rarely that every molecule of a dose of the liberator injected into an animal would be able to reach a suitable site of action and act with theoretical efficiency. But Compound 48/80 releases considerably more than this amount of histamine ; roughly, for each basic unit of its molecule it causes the appearance in the effluent from the perfused skin of 10–30 molecules of histamine. Even when simply injected intradermally into human skin, its effects were as great or greater than those of equal doses of histamine itself. The mechanism of histamine release by Compound 48/80, and presumably by all the other liberators which in other respects it so closely resembles, must therefore be one by which the liberator can cause the release of considerably more than its own molecular equivalent of histamine.

It is interesting that Compound 48/80, although itself inactive on the guinea-pig's gut, possesses the ability of antagonizing the contraction produced by the group of "slow-contracting substances," in small doses comparable to or less than those which affect the response to histamine and acetylcholine. Such an antagonistic action has not hitherto been described. The effect is hardly specific enough to be of much practical use, although it may help in defining some of the properties of these substances. It is of interest, however, in furnishing a common property between all the "slow-contracting substances" and strengthens the supposition that they are more or less closely related.

The relationship between Compound 48/80 and "slow-contracting substances" extends even further; for a substance of this type appears, after a dose of Compound 48/80, in relatively large quantity in the plasma of both cat and dog. The appearance of "slow-contracting substance" has been described before in experiments on the effect of venoms on various perfused organs (Feldberg, Holden, and Kellaway, 1938; Trethewie, 1939) and in anaphylaxis and peptone shock (Beraldo, 1950). In general it has been regarded as a side-product of the process of histamine release. But when it is found that a highly active histamine liberator not only causes the release of "slow-contracting substance" into the circulation, but bears so close a relationship to it as also to antagonize its effects, the possibility must now be considered that "slow-contracting substance" is itself directly involved in the histamine liberating reaction, although it is still too early to consider how this might come about.

The chemical structure of Compound 48/80 is unusual pharmacologically. The fact that it has proved to have such a high potency in one respect, that of histamine liberation, and that it displays a novel antagonism, that against "slow-contracting substances," suggests that a study of such basic "polymers" might be fruitful in yet other directions.

## SUMMARY

1. Compound 48/80, a condensation product of *p*-methoxyphenylethyl-methylamine and formaldehyde, injected intravenously in doses of 10 $\mu$g./kg. or more into the cat, causes a delayed depressor response. The depressor effect is associated with peripheral vasodilatation, and a rise in the haematocrit.

2. In the cat and the dog, intravenous injections of Compound 48/80 cause the release of histamine into the blood in amounts adequate to account for the depressor action of the drug. When a second dose fails to cause any further histamine release, there is also no depressor effect.

3. In the dog, Compound 48/80 causes a rise in portal pressure and a delay in the coagulation of the blood.

4. Compound 48/80 elicits a "triple response" from human skin when injected intradermally in a concentration of 1 : 1,000,000, and causes the appearance of large amounts of histamine in the venous effluent when injected into the isolated perfused skin preparation. A dose of 1 $\mu$g. Compound 48/80 caused the release of 7.7 $\mu$g. histamine base from this preparation.

5. After the injection of Compound 48/80, a "slow-contracting substance" appears in the plasma of both cat and dog. Compound 48/80 antagonizes the action of this substance on the guinea-pig's ileum, and also that of substance P,

bradykinin, and the "slow-contracting substance" present in extracts of dog and cat small intestine.

6. Compound 48/80 exerts a moderately strong antagonism to the effects of acetylcholine and histamine on the guinea-pig ileum.

I am greatly indebted to Dr. E. J. de Beer, the Wellcome Research Laboratories, Tuckahoe, N.Y., for generous supplies of Compound 48/80.

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# THE INFLUENCE OF AN ANTIHISTAMINE DRUG ON THE RELEASE OF HISTAMINE IN THE UNANAESTHETIZED DOG

BY

W. D. M. PATON AND M. SCHACHTER

*From the National Institute for Medical Research, Mill Hill, London, N.W.7*

(Received May 18, 1951)

There is evidence that the histamine content of the blood increases under a variety of conditions, including anaphylaxis, and after the administration of adrenaline, the wetting agent Tween 20, and a variety of organic bases (Feldberg, 1941 ; Krantz, Carr, Bird, and Cook, 1948 ; MacIntosh and Paton, 1949). Little information has been acquired, however, about the ability of the antihistamine compounds to influence the process of histamine release. Wells, Morris, and Dragstedt (1946), in only two experiments showed that, despite protection with benadryl, the anaphylactic reaction was accompanied by high levels of plasma histamine. This indicates that benadryl does not prevent the release of histamine in anaphylaxis. Staub (1946) claims, however, that the release of histamine into the blood after administration of adrenaline to man is prevented by antistin. Grossman and Robertson (1948), using the gastric secretion of the total stomach pouch of dogs as an index of released histamine, observed that the gastric secretion following administration of Tween 20 was greatly reduced by benadryl.

The interpretation of these results is difficult, particularly since neither antistin (Reuse, 1948) nor benadryl (Schild, 1947) is a specific antihistamine, and since the total pouch used by Grossman has unusual secretory characteristics (Woodward, Dragstedt, Tovee, Oberhelman, and Neal, 1945). So much importance, however, both theoretical and practical, attaches to any drug which can modify the process of histamine liberation that we have further examined the problem. As an index of histamine release we measured the hydrochloric acid secreted by the intact cannulated stomach of trained dogs. Mepyramine maleate (neoantergan) was selected as an antihistamine because of its high specificity (Schild, 1947 ; Reuse, 1948). The histamine liberator Compound 48/80, a condensation product of *p*-methoxyphenylethylmethylamine and formaldehyde (Baltzly, Buck, de Beer, and Webb, 1949 ; Paton, 1951), provided a simple means of obtaining a gradable histamine-releasing action without tissue destruction or signs other than those due to histamine release. In a few experiments, another histamine liberator, propamidine (MacIntosh and Paton, 1949) was used.

## METHODS

Four dogs, weighing between 10 and 20 kg., trained to stand quietly in a frame for hours, were equipped with gastric cannulae made of monel metal. Under these conditions the gastric reaction was always alkaline unless we deliberately took measures

TABLE I

GASTRIC SECRETION IN RESPONSE TO COMPOUND 48/80 WITH AND WITHOUT MEPYRAMINE

Compound 48/80, 10 mg. subcutaneously in all experiments. Italic figures refer to experiments in which 2 mg. mepyramine per kg. was given subcutaneously 15 min. before Compound 48/80

| Dog | c.c. 0.1N-HCl secreted on days: 1 | 3 | 10 | 12 | Ratio of secretion after 48/80 + mepyramine to secretion after 48/80 alone |
|---|---|---|---|---|---|
| Spot .. .. .. .. | *25* | 10 | 10 | *6* | 1.55 |
| Dusky .. .. .. | 60 | *124* | *106* | 14 | 3.11 |
| Jackie .. .. .. | *103* | 45 | 21 | *5* | 1.64 |
| Blackie .. .. .. | 32 | *37* | *40* | 19 | 1.52 |
| Total .. | 220 | 216 | 177 | 44 | Av. 1.95 |

to stimulate secretion. The amount of saliva collected from the cannula over a period of 1–2 hours is not more than a few c.c., which did not interfere with the "total" hydrochloric acid titrations, since phenolphthalein was used as indicator. Regurgitation of bile is a rare occurrence under these conditions. Compound 48/80 was given as the chloride, propamidine as isethionate, mepyramine as maleate, and histamine as acid phosphate. All drugs were administered subcutaneously. The thickness of the jowl of the upper jaw about 1–2 in. from the snout was measured in duplicate with a micrometer modified so that the jowl was enclosed between two flat surfaces during measurement. A lightly loaded spring ratchet enabled the reading to be taken with a reproducible gentle pressure, so that the maximum error with repeated measurement was 0.5 mm. In the experiments of Table II, the tests on each pair of dogs were crossed over, as indicated.

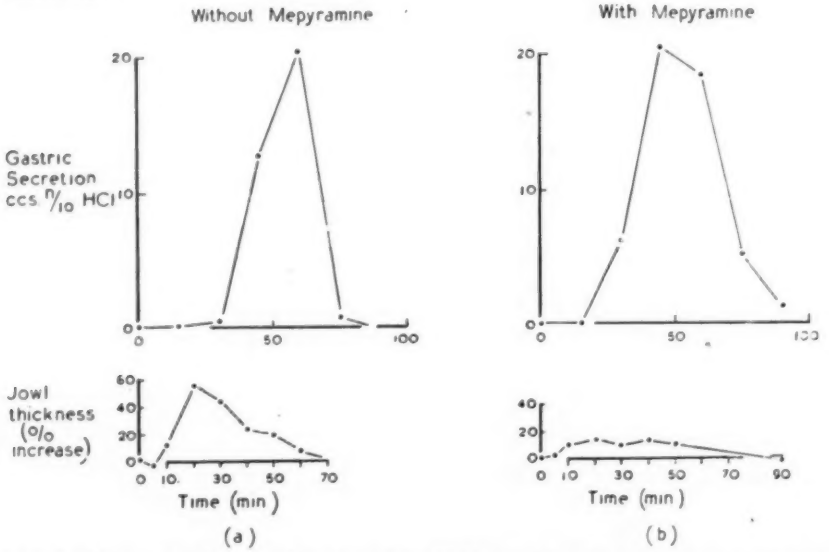


FIG. 1.—Dog Jackie. Gastric secretion (c.c. 0.1N-HCl per 15 min. sample: upper curves), and increase in jowl thickness (%: lower curves), in response (*a*) to 10 mg. Compound 48/80 subcutaneously at zero time, (*b*) to 10 mg. Compound 48/80 subcutaneously at zero time, with mepyramine 2 mg./kg. subcutaneously 15 min. previously. (*a*) and (*b*) are each the average of two crossed-over tests.

## RESULTS

The subcutaneous injection of 10 mg. Compound 48/80 into a dog always produced signs of severe itchiness, facial swelling, and secretion of gastric juice lasting 1–2 hours (Fig. 1). The facial swelling was particularly marked in the bristle area near the mouth, the eyelids, and the pinna of the ear; these areas also showed considerable erythema. Oedema and erythema of other regions was not observed, except for the nipple area in one animal. The gastric secretion produced by this dose of Compound 48/80 corresponded to that elicited by 1–2 mg. histamine acid phosphate.

Although the facial swelling and the signs of pruritus were almost entirely abolished by mepyramine, the gastric secretion was never diminished and usually considerably increased (Fig. 1, Table I). Further evidence that this dose of mepyramine was effective against either injected histamine or the circulatory depressor action of a liberator was that in the anaesthetized animal the depressor action of the liberator was reduced, and that in an unanaesthetized animal weal formation by intradermal injections of histamine was antagonized. It follows that an otherwise effective dose of mepyramine did not reduce the amount of histamine released by Compound 48/80.

The question now arises why the gastric secretion should actually be increased by mepyramine. The possibility that under these circumstances mepyramine alters the response of the gastric glands to histamine was excluded by demonstrating that in the same four dogs the gastric secretory response to injected histamine was not significantly altered by the same dose of mepyramine (Table II). Another explanation, however, may be that associated with the swelling and oedema of the face there is an oedema of the gastric mucosa, which is known to reduce gastric

TABLE II

EFFECT OF MEPYRAMINE ON GASTRIC SECRETION PRODUCED BY HISTAMINE

Histamine, 1 mg. subcutaneously. Mepyramine, 2 mg./kg. subcutaneously given 15 min. before histamine

| Dog | c.c. 0.1N-HCl produced by Histamine *a* | Histamine + Mepyramine *b* | Ratio *b*/*a* |
|---|---|---|---|
| Spot .. .. .. | 22.1 | 25.0 | 1.13 |
| Dusky .. .. .. | 34.4 | 23.1 | 0.67 |
| Jackie .. .. .. | 84.6 | 65.1 | 0.77 |
| Blackie .. .. .. | 38.0 | 51.4 | 1.35 |
| Total .. .. | 179.1 | 164.6 | Av. ratio = 1.02 |

secretion (Ricketts, Kirsner, and Palmer, 1949). The action of mepyramine in preventing this oedema would then allow the gastric secretion to proceed unimpeded during a period when it would otherwise be inhibited. Some support for this was obtained by the observation that the gastric mucosa was congested and oedematous to direct inspection in a dog anaesthetized after injection of Compound 48/80; also, in our unanaesthetized dogs, in the absence of mepyramine

the gastric secretion resulting from Compound 48/80 did not become maximal until the facial oedema began to decline.

Propamidine was tested in the same way, and it also produced gastric secretion, facial oedema (cf. Lourie and York, 1939), and itching; as with Compound 48/80, a vigorous gastric secretion was obtained in the presence of mepyramine. Similarly, gastric secretion in an anaesthetized animal following continuous infusion of propamidine was not significantly altered by mepyramine in the same dose. Propamidine, however, proved unsatisfactory for the purpose of this investigation, because after receiving several injections the animals gradually became moribund and had to be sacrificed. This drug was therefore used in only a few experiments.

*Development of refractoriness to compound 48/80*

With repeated injections of Compound 48/80, given at intervals of 2–7 days, the gastric secretory response progressively diminished in all animals (Table I). This decreasing effectiveness of Compound 48/80 was not due to a fall in sensitivity of the gastric glands to histamine, since histamine injected subcutaneously still elicited the usual secretion. A condition of complete refractoriness to Compound 48/80 was obtained by decreasing the interval between injections (Fig. 2). Such an animal not only shows no gastric secretory response to Compound 48/80, but

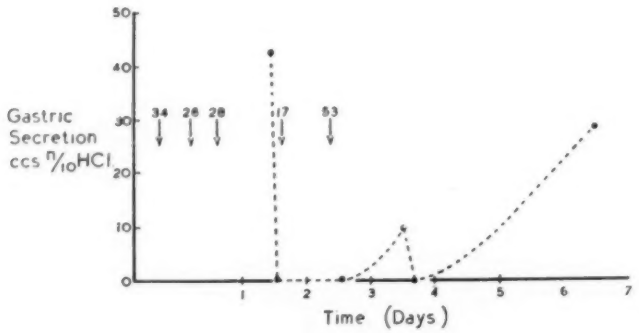


FIG. 2.—Graph of total gastric secretion (c.c. 0.1N-HCl) produced by repeated injections at varying intervals of 10 mg. Compound 48/80. Inserted figures are gastric secretory responses (c.c. 0.1N-HCl) to 1 mg. histamine acid phosphate at times marked by accompanying arrows.

facial swelling and signs of itchiness are also completely absent. This was repeatedly obtainable in two dogs in which it was attempted. This refractoriness does not, however, involve the secretory response to histamine; for instance in Fig. 2 on the second day a gastric secretion of 53 c.c. 0.1N-HCl was obtained with 1 mg. histamine acid phosphate, although immediately afterwards 10 mg. Compound 48/80 failed to elicit any secretion at all. Responsiveness to Compound 48/80, however, is partially restored after 1–2 days (Fig. 2).

## DISCUSSION

The inability of mepyramine to influence the actual release of histamine, while largely eliminating signs and symptoms due to injected or released histamine, is of interest for the interpretation of the mechanism of action of antihistamine drugs in clinical conditions where histamine release is involved. Our experiments suggest

that the therapeutic usefulness of these drugs under such circumstances may lie entirely in their ability to interfere with actions of histamine subsequent to its release, whereas the release itself is not interfered with.

The remarkable phenomenon of temporary complete refractoriness to a histamine liberator merits further investigation. For example, it would be of interest to know whether or not protection against a liberator such as Compound 48/80 confers resistance to other conditions associated with histamine release. In this way it might prove possible to determine whether histamine is liberated by different mechanisms under different conditions. Our experiments do not distinguish the possible processes involved in this refractoriness, such as the decrease in available histamine, a reduced " sensitivity " of the tissues, or the development of protective autacoid mechanisms.

## Summary

The histamine liberator Compound 48/80 is capable of releasing large amounts of histamine in the unanaesthetized dog. Although signs associated with histamine release, such as facial swelling and itchiness, are almost completely eliminated by mepyramine, the amount of histamine released, as determined by the amount of gastric secretion, is not reduced.

Repeated administration of Compound 48/80 produces a temporary state of complete refractoriness to the same dose of the drug.

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# THE INHIBITION OF SOME PHARMACOLOGICAL ACTIONS OF PENTAMIDINE BY SURAMIN

BY

J. L. GUIMARAES AND E. M. LOURIE

*From the Department of Pharmacology, University of Oxford*

(Received May 17, 1951)

When pentamidine and the other aromatic diamidines were first tested in man it was noted that, unlike earlier (and chemically quite unrelated) compounds used in the treatment of sleeping sickness, such as suramin and tryparsamide, they caused an immediate and profound, but transient, reaction on intravenous injection of a dose of therapeutic size. This consisted of a steep and sometimes alarming fall of blood pressure, often accompanied by intense itching, nausea, and colic, and occasionally by vomiting and defaecation. There was often also "a curious puffy suffusion of the face and eyelids" (Lourie, 1942). In laboratory animals Wien (1943) described a number of pharmacological effects, including fall of blood pressure (shown to be caused by peripheral vasodilatation) and stimulation of the isolated intestine of the rabbit and uterus of the guinea-pig and cat. The reactions in man and in animals were later, on sound evidence, attributed by MacIntosh and Paton (1949) to the liberation of histamine by the injected diamidine.

In the course of a "mass treatment" campaign against an epidemic outbreak of sleeping sickness in Sierra Leone in 1939 to 1941, several hundred patients were treated with pentamidine and other diamidines, and many more with suramin and tryparsamide (Lourie, 1942). In the early part of the work it was noticed on occasion that, when a syringe was filled with a 1 per cent aqueous solution of pentamidine dihydrochloride or one of the other diamidines, a fine flocculent precipitate would form in the solution welling into the barrel of the syringe. This was discovered to occur only when the syringe was one that had previously been used for an injection of suramin and that had then been imperfectly washed and sterilized, so that traces of suramin remained present. (The work was done in a remote part of the country, with primitive technical assistance or other facilities.) The precipitate is no doubt attributable to the formation of an insoluble salt complex between the diamidine, which is a strong base, and suramin, which is a polysulphonic acid derivative. Such an interpretation accords well with what is known about the physicochemical reactivity of unsubstituted amidines with sulphonic and other acids (Walker, 1949). Salt- and precipitate-formation similarly occur when mixtures are made of solutions of curare and congo red or related polysulphonated substances (Petroff, 1931). This has recently been re-examined in respect of pure *d*-tubocurarine chloride by Kensler (1949).

It occurred to us that the formation of such a pentamidine-suramin salt complex might impair the pharmacological properties of one or other of the two compounds concerned, and the experiments below were designed to test this possibility. It has, in fact, been shown by Petroff (1924), Kensler (1949) and others that congo red and related substances do inhibit some of the characteristic pharmacological properties of curare. This inhibition was ascribed by Kensler to the reaction which takes place between the two compounds concerned, rather than to an intrinsic effect of congo red or other sulphonated compounds on the tissue sites to which *d*-tubocurarine is adapted. It is in this way also that we would explain the inhibition of some of the pharmacological actions of pentamidine by suramin in the experiments described below.

The actual formation of a precipitate is, of course, not a necessary accompaniment of salt formation (and hence of the possibility of inhibitory properties) between compounds of the two types concerned. This is true not only for concentrations below the thresholds at which precipitation occurs but also at concentrations considerably above those thresholds. As Kensler pointed out, the precipitate formed by congo red and tubocurarine can be dissolved in excess congo red. In the same way we have found that the precipitate formed by suramin and pentamidine can be dissolved in excess suramin. Thus in a series of suramin-pentamidine isethionate mixtures in distilled water where the pentamidine-concentration is constant at 1/100 (the concentration that was usually used for the injections in man) and suramin is present in increasing concentrations, a faint opalescent precipitate appears at about 1/100,000 suramin and becomes dense and flocculent from 1/2,000 to 1/25, but clears up completely at 1/10. If the pentamidine concentration be kept constant at 1/1,000 the dense precipitate present at the lower concentrations of suramin clears up when this approaches 1/50. If the pentamidine be kept at 1/10,000 no concentration of suramin causes a heavy precipitate, and the light precipitate found at lower concentrations of suramin clears up when this exceeds about 1/1,000.

### *Fall of blood pressure*

Table I and Figs. 1 and 2 show the effects of an intravenous injection of pentamidine isethionate on the systolic carotid blood pressure in 16 cats (under chloralose), 8 of which served as controls (i.e., not previously treated with suramin), and the other 8 had previously received an intravenous injection of suramin. The pentamidine dose was usually 1 mg./kg. but ranged up to 2.0 mg. in the few instances shown on the Table, and the suramin ranged from 50 to 250 mg./kg. The blood pressures recorded on the Table are, on the one hand, those immediately before the pentamidine injection and, on the other, the lowest reached within the next five minutes. The initial blood pressures in some of the cats were rather low; this was because those particular cats were used after they had already served for various other experiments, which were, however, of a nature unlikely to affect the present points at issue. The prior dose of suramin sometimes produced a transient fall of blood pressure, or a slow fall returning within 15 minutes to about or near the original level, but the pentamidine was not given until a steady level had been regained. The interval between the injections of suramin and pentamidine varied between five and eighty minutes. Two of the cats that received suramin followed by pentamidine (Exps. 11 and 14) had already received a dose of pentamidine before

TABLE I

FALL OF CAROTID BLOOD-PRESSURE IN CHLORALOSED CATS AFTER INTRAVENOUS PENTAMIDINE, AND ITS INHIBITION BY INTRAVENOUS SURAMIN. PRESSURES RECORDED ARE THOSE IMMEDIATELY BEFORE PENTAMIDINE AND THE LOWEST REACHED WITHIN THE NEXT FIVE MINUTES

| Pentamidine alone | | | | Suramin followed by pentamidine | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Exp. | Pentam. dose (mg./kg.) | Blood pressure | | Exp. | Suramin dose (mg./kg.) | Interval between suramin and pentam. (min.) | Pentam. dose (mg./kg.) | Blood pressure | |
| | | Before pentam. (mm.Hg) | After pentam. (mm.Hg) | | | | | Before pentam. (mm.Hg) | After pentam. (mm.Hg) |
| 5 | 2.0 | 33 | 10 | 1 | 200 | 80 | 1.2 | 93 | 93 |
| 6 | 1.0 | 56 | 34 | 2 | 100 | 15 | 1.0 | 35 | 29 |
| 7 | 1.0 | 33 | 20 | 3 | 100 | 45 | 1.7 | 40 | 40 |
| 10 | 1.0 | 145 | 71 | 4 | 50 | 20 | 2.0 | 65 | 57 |
| 12 | 1.0 | 58 | 20 | 8 | 250 | 40 | 1.0 | 96 | 103 |
| 13 | 1.0 | 128 | 60 | 9 | 250 | 35 | 1.0 | 91 | 83 |
| 15 | 1.0 | 87 | 51 | 11* | 100 | 5 | 1.0 | 107 | 107 |
| 16 | 1.0 | 125 | 79 | 14* | 100 | 8 | 1.0 | 110 | 110 |
| | Averages | 83 | 43 | | | | Averages | 80 | 78 |

* One injection of pentamidine already given before the suramin in this experiment.

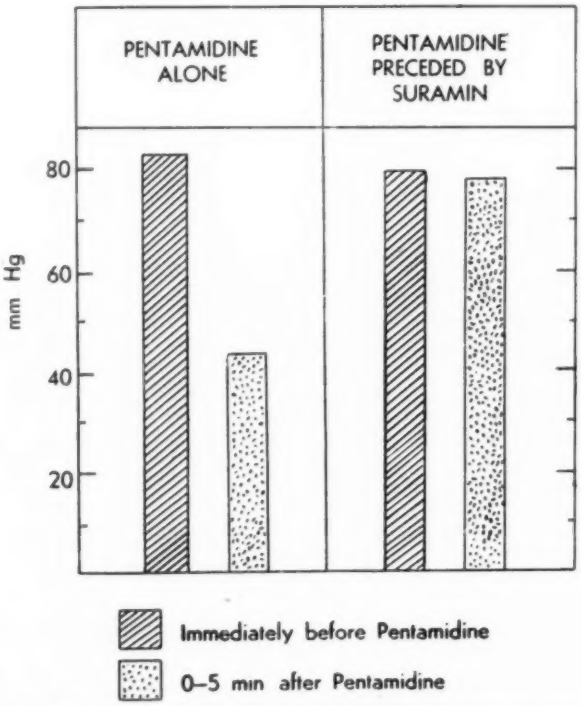


FIG. 1.—Inhibitory effect of suramin on the fall of carotid blood pressure caused by pentamidine. Average pressures in cats (chloralose) immediately before and within 5 minutes after an intravenous injection of pentamidine; 8 cats received pentamidine alone, and 8 received pentamidine preceded by suramin (see Table I).

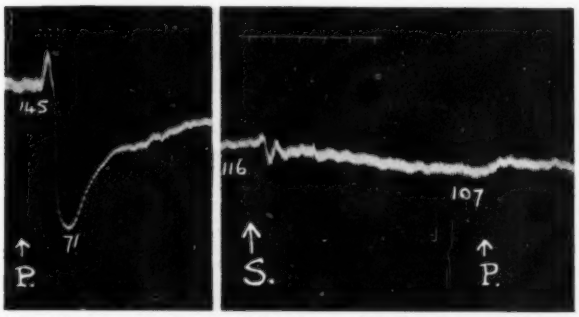


FIG. 2.—Inhibitory effect of suramin on the fall of carotid blood pressure caused by pentamidine. Cats of exps. 10 and 11, Table I. P. = Pentamidine, 1.0 mg./kg. i.v. S. = Suramin, 100 mg./kg. i.v. 30-second time-intervals.

the suramin. In other experiments it was noted that repeated injections of pentamidine usually produced progressively smaller depressor responses (see also Wien, 1943). In the two cats of Exps. 11 and 14, therefore, the inhibitory effect of suramin on the pentamidine-response might have been reinforced by the earlier injections of pentamidine. However, an inhibitor effect of suramin in these two instances remains beyond doubt, since a second injection of pentamidine normally produces merely a smaller response and not the complete abolition observed in the two cases in question. The Table and Fig. 1 show that in the cats that had previously received no suramin the blood pressure was reduced by an injection of pentamidine from an average of 83 to 43 mm. Hg, while in the suramin-treated cats the fall caused by pentamidine was negligible: Fig. 2 shows tracings from two representative experiments (Nos. 10 and 11) of the series.

*Broncho-constriction*

In view of the known pharmacological actions of pentamidine, to be regarded as the effects of histamine release, some evidence of broncho-constriction is to be expected. This can readily be shown in guinea-pigs by the technique of Konsett and Rössler (1940). Fig. 3 (guinea-pig *a*) represents a typical broncho-constrictor

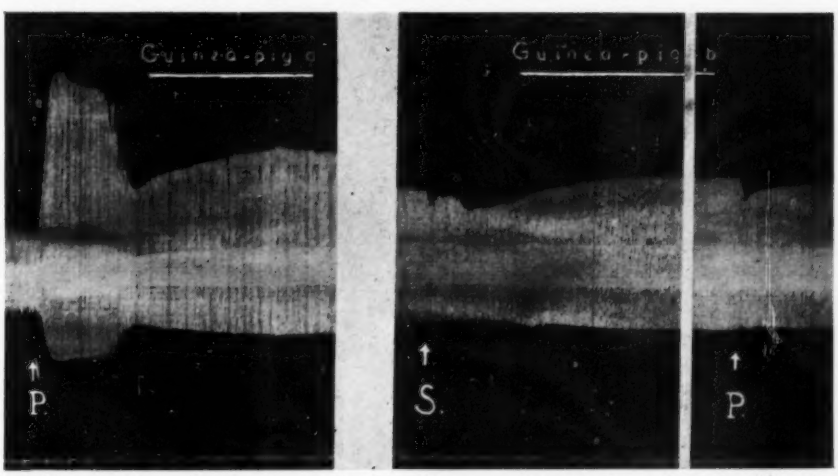


FIG. 3.—Inhibitory effect of suramin on the broncho-constrictor action of pentamidine in guinea-pigs. Urethane anaesthesia. P. = Pentamidine, 25 mg./kg. i.v. S. = Suramin, 250 mg./kg. i.v. 60-second time-intervals. Break in the tracing for guinea-pig *b* represents 20 minutes.

effect, as shown by this method, after the intravenous injection of 25 mg./kg. pentamidine isethionate in a guinea-pig anaesthetized with urethane. A smaller dose does not regularly produce the effect. The reaction begins about a minute after injection but is sometimes delayed for two or even three minutes (a latent period which, in respect of the fall of blood pressure, was stressed by MacIntosh and Paton as probably significant of the underlying mechanism of histamine release). The constrictor response reaches its maximum a few minutes after its onset, and there is then a gradual relaxation, though there is often (as in guinea-pig *a*, Fig. 3) a secondary low-grade constriction lasting for perhaps a quarter of an hour before a stable condition is reached. In numerous experiments we have found that the constrictor action of 25 mg./kg. pentamidine may be aborted by a prior intravenous injection of 250 mg./kg. suramin, and this is shown in the second portion of Fig. 3 (guinea-pig *b*), in which the pentamidine was given 20 minutes after the suramin. In order that the inhibition of the pentamidine-effect by suramin may be attributed with assurance to the latter drug, it is advisable to use a guinea-pig that has had no dose of pentamidine previous to the suramin, since it has been found that a pig that has had one injection of 25 mg./kg. pentamidine may fail completely to respond to a second such injection. It is for this reason that Fig. 3, illustrating inhibition of the broncho-constrictor effect of pentamidine, is derived from observations on two animals, while Fig. 1, dealing with inhibition of the fall of blood pressure, is derived from observations on a single animal. In other experiments we have found, as would be expected, that a suitable dose of an antihistamine compound (e.g., 2.5 mg./kg. mepyramine) is also capable of completely aborting the broncho-constrictor effect of an injection of 25 mg./kg. pentamidine, a result which supports the view that histamine release accounts for at least some of the pharmacological effects of pentamidine with which we are here concerned.

We were interested in comparing the picture produced by the Konsett and Rössler apparatus after an injection of pentamidine with the picture produced by acetylcholine. In the course of these observations an unexpected finding emerged. This was that the tracing for a dose of acetylcholine given before a guinea-pig had received any injection of pentamidine was very different from the tracing produced by the same dose of acetylcholine after an injection of pentamidine (and after time had been allowed for the broncho-constrictor effect of the pentamidine to subside). This is illustrated by Fig. 4, guinea-pig *a*, where the break in the tracing represents a period of 30 minutes, during which a dose of 25 mg./kg. pentamidine was allowed to exercise its effect. Clearly the action of acetylcholine was greatly potentiated by the interposed dose of pentamidine. An explanation of this potentiation was sought in the possibility that there might be a slow release of histamine continuing for some time after the pentamidine injection, this slow release being itself insufficient to cause broncho-constriction but having the effect of exaggerating the response to a dose of acetylcholine injected during the period of release. The explanation was tested indirectly in guinea-pig *b* of Fig. 4 by slowly infusing histamine intravenously at the rate of 1$\mu$g. per minute, which was insufficient to cause any constrictor action, and comparing the effect produced by a dose of acetylcholine injected during this infusion with the effect produced by the same dose of acetylcholine before the infusion had begun. The slow histamine infusion did indeed have the effect of potentiating the guinea-pig's response to acetylcholine,

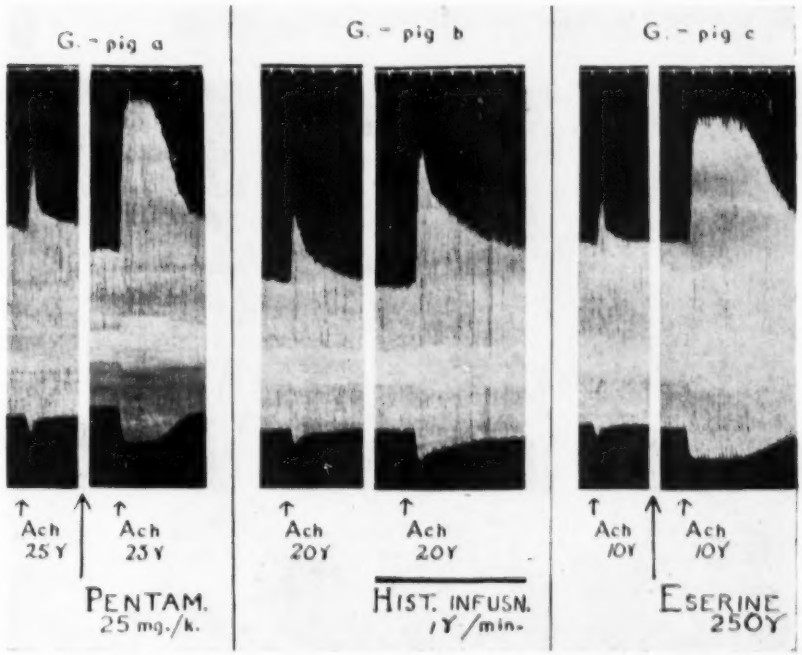


FIG. 4.—Broncho-constrictor response to acetylcholine in guinea-pigs. Potentiation caused by pentamidine (guinea-pig *a*), histamine infusion (guinea-pig *b*), and eserine (guinea-pig *c*). Urethane anaesthesia. Ach. = Acetylcholine. Pentam. = Pentamidine. Hist. infusn. = Histamine infusion. All injections and the infusion i.v. 60-second time-intervals. Break in the tracing for each guinea-pig represents 30 minutes.

but there is an important difference between the tracings for acetylcholine potentiation attributable to the histamine infusion (guinea-pig *b*) and the potentiation provoked by a prior dose of pentamidine (guinea-pig *a*). This is that during the histamine infusion the characteristic shape of the tracing normally produced by acetylcholine is unimpaired ; the band immediately widens and forthwith begins to return again to its original state, giving the appearance which, for convenience, we call a " peak " effect (guinea-pig *b*). After a dose of pentamidine, on the other hand, the band again immediately widens in response to an injection of acetylcholine, but it then remains at the maximum width for a relatively long time, which may be as much as two or three minutes, before the effect begins gradually to subside. For convenience, we call this a " broad-end " effect (second part of tracing for guinea-pig *a*). It is clear, therefore, that the exaggerated response to acetylcholine in an animal that has previously received a dose of pentamidine is unlikely to be explained merely as the effect of acetylcholine injected during a period of slow histamine release. An alternative explanation is strongly suggested by the shape of the broad-end tracing, since this indicates not only a greater degree of broncho-constriction but also a significant persistence of the effect. The alternative explanation is that the potentiation is due to an anticholinesterase effect of the prior dose of pentamidine. Anticholinesterase activity has been demonstrated in the closely related diamidine, stilbamidine, by Bergmann, Wilson, and Nachmansohn (1950). That this is the likely explanation is supported by showing that if a dose of eserine is interposed between two injections of acetylcholine, the response is converted

from the peak to the broad-end type in the same way as when a dose of pentamidine has been interposed (guinea-pig *c*, compared with guinea-pig *a*, Fig. 4).

Since an anticholinesterase property of pentamidine is held to be responsible for changing the acetylcholine-response from the peak to the broad-end type, the question arises whether the known antihistaminase property of pentamidine (Blaschko and Duthie, 1944) is not capable of bringing about a similar change in the response of the bronchi to histamine. Fig. 5 shows that this is so, and it therefore provides

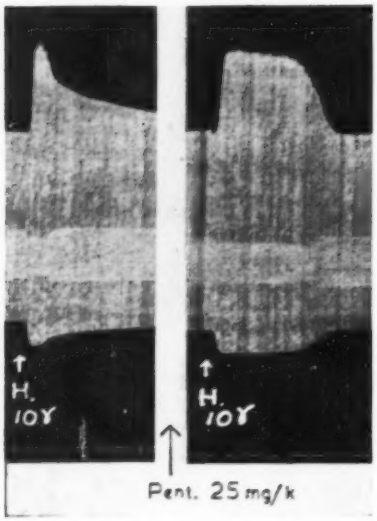


FIG. 5.—Potentiating effect of pentamidine on broncho-constrictor response of guinea-pigs to histamine. Urethane anaesthesia. H. = Histamine. Pent. = Pentamidine. 60-second time-intervals. Break in the tracing represents 30 minutes.

circumstantial evidence of antihistaminase activity on the part of pentamidine. Can similar circumstantial evidence be obtained in favour of an antihistaminase property on the part of suramin as well ? This compound is already known to be capable of inhibiting a wide range of other enzyme systems (Town, Wills, Wilson, and Wormall, 1950 ; Wills and Wormall, 1950). If an injection of suramin were to exercise any antihistaminase activity, then the ability of such an injection to inhibit the broncho-constrictor effect of a subsequent dose of pentamidine must represent a high inhibitory power indeed ; for the antihistaminase property of the suramin would tend to potentiate rather than to inhibit the pentamidine response (i.e., the response to liberated histamine). If, on the other hand, suramin has the property of inhibiting and not of potentiating the effects of a dose of histamine, then this antihistamine action might alone be sufficient to account for suramin's inhibitory effect on pentamidine, without the formation of a salt complex playing any essential part in the inhibitory mechanism. It is therefore important to determine the influence which suramin exercises on the response of guinea-pig bronchi to an injection of histamine.

Fig. 6 is a tracing from one of many experiments which showed that suramin has, in fact, some inhibitory action on histamine. The broncho-constrictor effect of 4 μg. histamine was abolished by an injection of 250 mg./kg. suramin, and it was necessary to give 16 μg. histamine after the suramin in order to produce about the same degree of constriction as was caused by one quarter of that dose before the

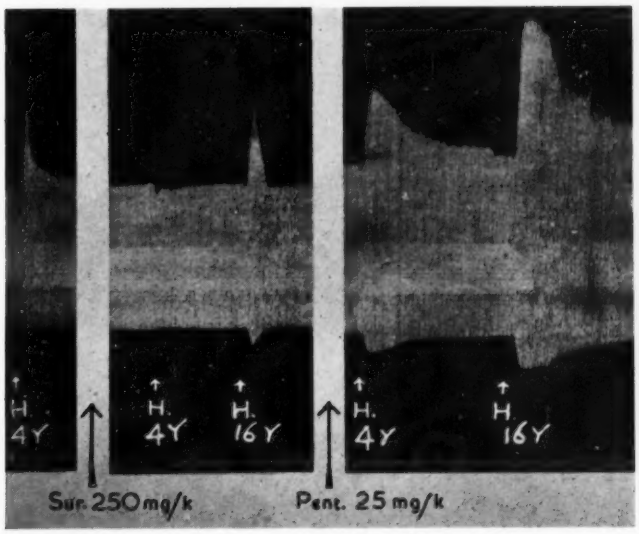


FIG. 6.—Antihistamine effect of suramin on guinea-pig bronchioles, and counteraction of this effect by the potentiating influence of pentamidine. Urethane anaesthesia. H. = Histamine. Sur. = Suramin. Pent. = Pentamidine. 60-second time-interval. Breaks in the tracing represent intervals of 20 and 10 minutes.

suramin. The question therefore becomes acute whether this antihistamine action of suramin can indeed account for its inhibitory effect on pentamidine. This seems unlikely, because Fig. 6 also shows that the antihistaminase action of the pentamidine dose more than counteracted the antihistamine action of the suramin, so that the response to 4 μg. histamine, which had been abolished by the suramin, was now greater than before. The anti-pentamidine action of suramin is therefore unlikely to be fully explained by suramin's antihistamine property, and inactivation of the pentamidine by formation of a suramin-pentamidine salt complex remains a tenable explanation.

It was found, incidentally, that the antihistamine action of suramin is non-specific. An inhibitory action of the same general order is exercised also against acetylcholine. For example, in one typical experiment the broncho-constrictor effect of 5 μg. acetylcholine was abolished by 250 μg. suramin, and it was necessary to give five times that amount of acetylcholine in order to produce about the same effect as before the suramin.

*Contraction of isolated gut*

As a preliminary to investigating the inhibitory effect of suramin on the power of pentamidine to cause contraction of the isolated gut, we set out to confirm that suramin has an antihistamine effect on this preparation.

With a piece of guinea-pig ileum suspended in oxygenated Locke solution at 32° C., it was found that suramin in a concentration of 1/1,000 caused tonic and spasmodic contractions ; concentrations of 1/1,000 to 1/5,000 caused in some experiments occasional spasmodic contractions during only about the first 10 minutes of exposure, and concentrations below 1/5,000 had little or no visible effect. Observations were then made on the response of guinea-pig ileum to a uniform dose of

histamine introduced at 5- or 10-minute intervals (*a*) before the addition of suramin to the surrounding Locke solution; (*b*) in the presence of varying concentrations of suramin; and (*c*) in the absence of suramin but after the gut had been exposed to that substance for varying lengths of time. The method used and results obtained are sufficiently shown on Table II and Figs. 7 and 8, from which it will be seen

TABLE II

SUMMARY OF EXPERIMENTS TO SHOW INFLUENCE OF SURAMIN ON THE RESPONSE OF ISOLATED GUINEA-PIG ILEUM TO HISTAMINE

| Suramin concentration | No. of exps. | Frequency of histamine test* | Duration of contact with suramin (in min. in respective experiments) | Histamine response, in relation to response before contact with suramin | |
|---|---|---|---|---|---|
| | | | | During contact with suramin | After contact with suramin |
| >1/10,000 .. | 3 | 5 min. | 10, 30, 30 | Potentiation | Inhibition |
| | 1† | 10 min. | 60 | Potentiation | Inhibition |
| 1/10,000–1/25,000 | 5 | 5 min. | 30, 30, 35, 40, 60 | Potentiation | Inhibition |
| | 2‡ | 10 min. | 50, 80 | Inhibition§ | Inhibition |
| 1/40,000–1/100,000 | 7 | 5 min. | 30, 30, 30, 30, 60, 75, 90 | No change‖ | No change¶ |
| | 2 | 10 min. | 50, 120 | No change | No change |

* Histamine dosage (chosen to give sub-maximal response at the beginning of the experiment) kept constant throughout each experiment; usually such that concentration in the bath was $10^{-8}$. Replaced by Locke or suramin-Locke solution as soon as full response had been produced.

† See Fig. 7.

‡ See Fig. 8.

§ Appeared in 10–20 and 20–30 minutes, respectively.

‖ Except in one experiment with 1/40,000 suramin, in which there was potentiation.

¶ Except in one experiment with 1/80,000, in which there was inhibition.

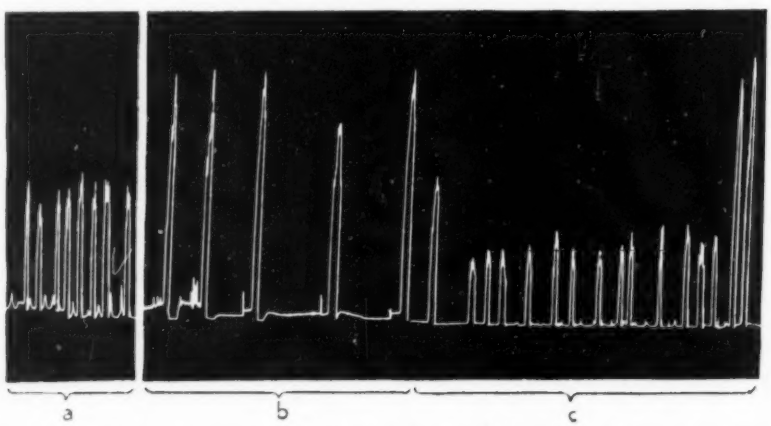


FIG. 7.—Potentiation of the response of isolated guinea-pig ileum to histamine in the presence of suramin and inhibition after its removal. Histamine $10^{-8}$ tested every 10 minutes in oxygenated Locke solution at 32° C. (*a*) before adding suramin to the Locke solution, (*b*) in the presence of 1/4,000 suramin, and (*c*) after replacing the suramin-Locke by normal Locke solution; last 2 contractions are in response to $5 \times 10^{-8}$ histamine.

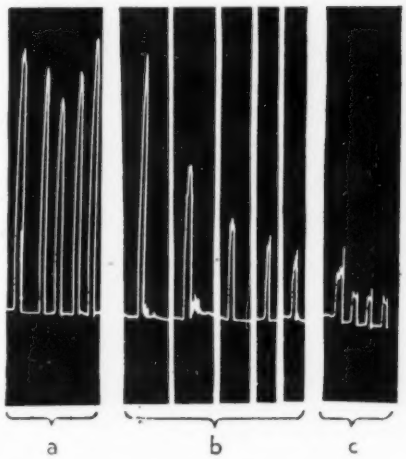


FIG. 8.—Inhibition of the response of isolated guinea-pig ileum to histamine both in the presence of suramin and after its removal. Histamine $10^{-8}$ tested every 10 minutes in oxygenated Locke solution at 32° C. (*a*) before adding suramin to the Locke solution, (*b*) in the presence of 1/10,000 suramin, and (*c*) after replacing the suramin-Locke by normal Locke solution. Section *b* is interrupted because the drum was not stopped between tests in this part of the experiment.

that suramin at concentrations above 1/10,000 in the fluid surrounding the gut serves to potentiate its response to histamine, but as soon as suramin ceases to be present in the surrounding fluid the histamine-response becomes inhibited (in relation to the effect observed before suramin was present). This inhibition persists for periods of from 20 minutes to at least 70 minutes (the limit of the observation-period), depending on the concentration of suramin that had been used and the duration of exposure. When the surrounding Locke solution contains suramin in concentrations of 1/25,000 to 1/10,000 there may again be potentiation of the histamine-response, followed by inhibition in suramin-free Locke solution. But the potentiation in the presence of suramin at these concentrations was observed only in those experiments (five in number) in which the response to histamine was tested every five minutes. In the two experiments in which the histamine-response was tested every 10 minutes the effect observed in the presence of suramin was inhibition instead of potentiation. Whether any significance attaches to the fact that the one effect was observed when the histamine-response was tested every five minutes and the other when it was tested every 10 minutes can be decided only by further experiment. When the suramin concentrations used were below 1/25,000 there was usually no observed change in the histamine-response either in the presence of suramin or after its removal.

A reasonable interpretation of these findings is that within a narrow concentration-range suramin does exercise an inhibitory action on the response of the isolated gut to histamine. While this is clear from some of the above experiments, the conditions necessary for its consistent demonstration remain to be accurately determined. In the presence of higher concentrations the tissue becomes abnormally excitable and its reactivity to histamine is enhanced. (It is possible that this hyper-excitability may be due to impurities rather than to the suramin itself; "Antrypol" brand, as commercially supplied by I.C.I., was used throughout.) During exposure of the gut to suramin, especially at the higher concentrations, some of the substance is adsorbed by the tissue cells and, depending on the actual concentration used and the duration of exposure, sufficient is retained for the cells to remain in the inhibitory range of suramin-concentration for a long time after the surrounding fluid has ceased to

contain any of the drug. This is in accordance with the well-established fact that suramin is adsorbed and retained by body tissues for long periods of time (Dewey and Wormall, 1946).

In order to demonstrate an inhibitory effect of suramin on the isolated gut we chose to use rabbit duodenum rather than guinea-pig ileum, because of the statement by Wien (1943) that rabbit gut is consistently stimulated by pentamidine at concentrations of from 1/25,000 to 1/10,000, while no marked effect is exerted on isolated guinea-pig ileum. We found that isolated rabbit duodenum in oxygenated Locke solution at 35° C. will respond repeatedly to successive doses of pentamidine at a concentration in the bath of 1/25,000. Fig. 9 shows the tracing from one experiment in which nine consecutive contractions were produced in this way. We have not resolved the question how far this ability of the gut to respond to repeated doses of pentamidine is consistent with histamine release as an explana-

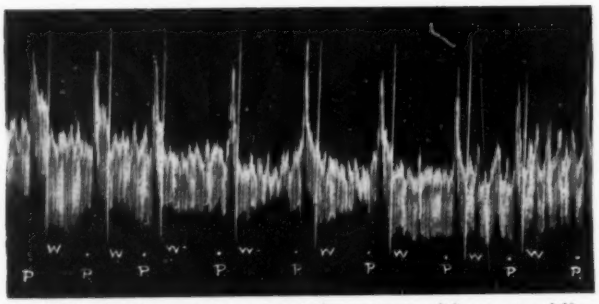


FIG. 9.—Response of rabbit duodenum to repeated contact with pentamidine isethionate in oxygenated Locke solution at 35° C. P. = Pentamidine 1/25,000. W. = Wash.

tion of the underlying mechanism of the stimulant action of pentamidine on the gut. In this connexion, though, an antihistamine (mepyramine) at a concentration of 1/100,000 can be shown to prevent the response normally caused by 1/25,000 pentamidine.

Fig. 10 also shows consecutive pentamidine responses, of rather greater degree than those of Fig. 9, and, on another piece of gut, abolition of the response to this

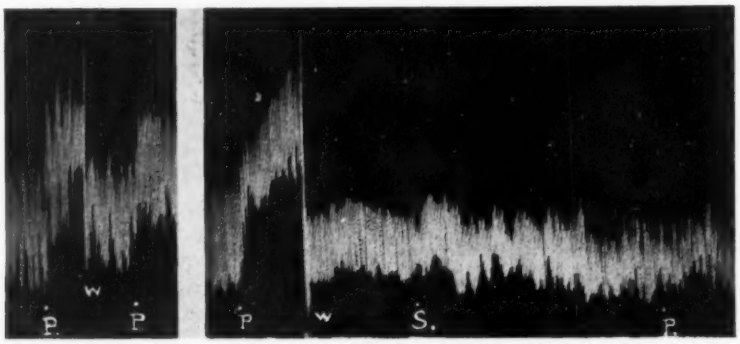


FIG. 10.—Inhibitory effect of suramin on the stimulant action of pentamidine on isolated rabbit duodenum in Locke solution at 35° C. P. = Pentamidine 1/25,000. S. = Suramin 1/5,000. W. = Wash.

concentration of pentamidine (1/25,000) by the presence in the Locke solution of suramin at a concentration of 1/5,000. In view of the possible role of histamine release in the mechanism of pentamidine activity on the gut, and the antihistamine action of suramin demonstrated in the experiments above, we cannot exclude the possibility that suramin's anti-pentamidine action on the gut may be partly due to antihistamine activity as well as to the formation of an inactive salt complex.

Changes in hydrogen ion concentration cannot be held to account for the effects on the histamine- or the pentamidine-response that we have attributed to suramin. Using a Muirhead *p*H-Meter (type D-303-B) we found that changes in the hydrogen ion concentration of Locke solution caused by the presence of up to 1/200 suramin are negligible.

### *Curare-like action**

*Depression of neuro-muscular transmission.*—Bergmann *et al.* (1950) claimed that stilbamidine blocks neuro-muscular transmission in the frog sciatic nerve gastrocnemius preparation. This led us to seek an action that might possibly be interpreted in this way on the rat phrenic nerve diaphragm preparation of Bülbring (1946). Fig. 11 shows the results of a typical experiment in which the preparation was set up in double dextrose Tyrode solution at 37° C. and single shocks of 0.73 mv. for 0.8 msec. were applied at 10-second intervals by means of a square-wave stimulator. Pentamidine in a concentration of 1/10,000 caused a slight temporary inhibition of the muscle contractions. The response to such a dose is repeatable a number of times in a single preparation, and in the experiment of Fig. 11 it was obtained six times, at intervals of about ten minutes, immediately before making the observations recorded on the Figure. It will be seen that the presence of $10^{-4}$ suramin in the double dextrose Tyrode solution used completely blocked the pentamidine effect, which could then be elicited again after washing. A slight precipitate formed in the bath while suramin and pentamidine were both present. In order to eliminate the possibility that the inhibition might be due simply to effective amounts of pentamidine being withdrawn from solution by the precipitate, similar observations were made using an excess of suramin (1/200) so that there should

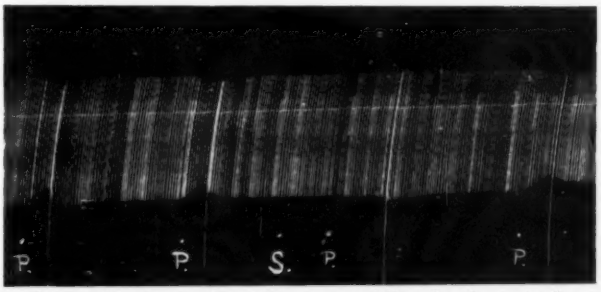


FIG. 11.—Depression of muscle contractions by pentamidine in the rat phrenic nerve diaphragm preparation, and abolition of this effect in the presence of suramin. Oxygenated double dextrose Tyrode solution at 37° C. P. = Pentamidine 1/25,000. S. = Suramin 1/10,000.

* The term curare-like is used here very loosely and without implying that the mechanism of action of pentamidine is exactly the same as that of curare.

be no actual precipitate present in the suramin-pentamidine mixture. In this concentration of suramin (before adding pentamidine) the contractions became somewhat greater than before. On the addition of pentamidine to give a concentration of 1/10,000, the suramin excess prevents the formation of any precipitate (or if formed it immediately goes into solution) and the inhibitory action of pentamidine on the contractions is abolished in the same way as when weaker (precipitate-forming) concentrations of suramin are used. At such a high concentration of suramin, however, the interference with pentamidine activity is much longer-lasting. In one experiment it persisted after eight Tyrode washings during a period of about 80 minutes, as tested by the introduction of pentamidine at a concentration of 1/10,000 shortly before each wash. That the ability of the preparation to respond to pentamidine was, however, not completely abolished was shown by inhibition of the muscle contractions when the pentamidine test-dose was increased to give a concentration of 1/2,000 in the bath. Persistence of the suramin-effect long after the surrounding fluid has been replaced by suramin-free Tyrode solution is not surprising in view of what is known about the power of tissue cells to adsorb and retain suramin, to which reference was made in the section above on contraction of isolated gut.

*Paralysis in frogs.*—The demonstration that pentamidine blocks neuro-muscular transmission on the phrenic nerve diaphragm preparation leads naturally to a consideration of other properties that this compound may have in common with curare. Its ability to paralyse frogs when injected into the ventral lymph sac was therefore investigated, together with the question whether this paralysis could be inhibited by suramin.

TABLE III

Inhibitory effect of suramin on pentamidine-induced paralysis in frogs. Both drugs injected into the ventral lymph sac. Suramin-treated frogs received only one dose of suramin, at the beginning of the experiment, but pentamidine was given daily to all frogs as long as they survived

| Exp. | Frog serial numbers | Number of frogs paralysed, in relation to the number treated | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1st day | | 2nd day | | 3rd day | | 4th day | |
| | | Drug and dose* | Result | Drug and dose* | Result | Drug and dose* | Result | Drug and dose* | Result |
| 1 | 1–6 | P. 0.75 | 4/6 | P. 1.5 | 6/ | P. 1.5 | 2/2 | P. 1.5 | 1/1 |
| | 7–12 | S. 20 + P. 0.75 | 0/6 | P. 1.5 | 1/5 | P. 1.5 | 0/2 | No survivors | |
| 2 | 13–18 | P. 1.5 | 6/6 | P. 1.5 | 3/3 | P. 1.5 | 1/1 | No survivors | |
| | 19–24 | S. 40 + P. 1.5 | 0/6 | P. 1.5 | 0/1 | P. 1.5 | 0/1 | No survivors | |

* P. = Pentamidine. S. = Suramin. · Dose in mg./20 g.

The injections into the ventral lymph sac were made in aqueous solution in 0.5 ml. per 20 g. body weight. Suitable doses of pentamidine were found regularly to cause a generalized flaccid paralysis which, if the frog recovered at all, lasted for two to five hours. Suramin was injected 10 minutes before the pentamidine, and the paralysis associated with the latter drug could thus be aborted; for the demonstration of this inhibition the suramin dose should not exceed 40 mg./kg., since larger doses in the frog cause a generalized spastic or tetanic condition which obscures any possible inhibitory influence that may be exerted on the effects of a subsequent pentamidine injection. Following Kensler (1949), paralysis was defined as inability of the frog to right itself five times consecutively when placed on its back, and Table III records the results of two experiments from which the following conclusions were drawn:

1. Pentamidine in a dose of 0.75 mg./20 g. usually or often paralyses, and a dose of 1.5 mg./20 g. regularly does so. The larger dose produced this effect on each of the nineteen occasions on which it was tested (without prior suramin).

2. The larger dose (1.5 mg./20 g.) may eventually kill the frog (after it has recovered from the paralysis), though some frogs will support repeated injections at 24-hour intervals for two or three days.

3. A prior injection of suramin in a dose of 20 or 40 mg./20 g. can protect a frog against the paralysing action of 1.5 mg./20 g. pentamidine.

4. This protective effect of a single injection of suramin may last for at least 48 hours.

### *Toxicity for mice*

An intraperitoneal injection of 2.75 or 3.25 mg./20 g. pentamidine isethionate is normally lethal to mice within 48 hours of treatment. After the larger dose (3.25 mg.) most of the deaths occur within six hours of the injection, but if

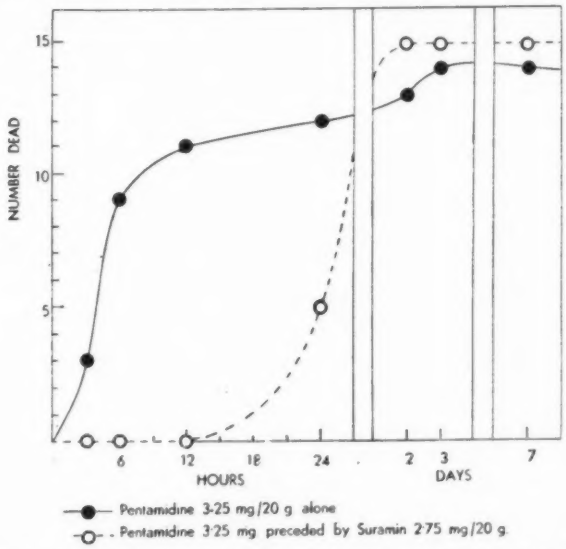


FIG. 12.—Delaying effect of suramin on the lethal action of pentamidine in mice. Intraperitoneal injections. Suramin half-an-hour before the pentamidine. Fifteen mice in each group.

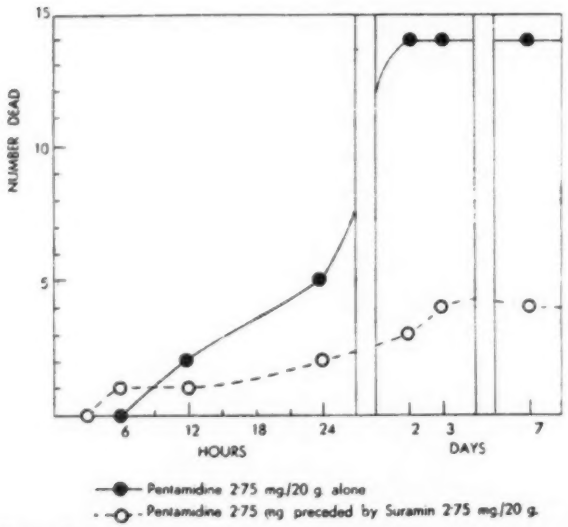


FIG. 13.—Inhibitory effect of suramin on the lethal action of pentamidine in mice. Intraperitoneal injections. Suramin half-an-hour before the pentamidine. Fifteen mice in each group.

2.75 mg./20 g. suramin be injected intraperitoneally half-an-hour before the pentamidine, the toxic action of the latter is delayed, so that most of the mice do not die until more than 24 hours later. This is shown in Fig. 12. Against the smaller dose of pentamidine (2.75 mg.) the protective action of 2.75 mg. suramin is, of course, less severely tested, so that most of the mice recover completely. This is shown in Fig. 13.

The question of suramin's antihistamine action, exercised against histamine released by the pentamidine injected, may be dismissed in explaining the inhibitory effect of suramin on pentamidine in mice and frogs, because of the relative insensitivity of these animals to histamine. Also, in frogs the effects observed were in no way to be associated with the known actions of histamine.

### *The question of chemotherapeutic interference*

Both pentamidine and suramin are extensively used in the treatment of sleeping sickness. It is therefore not only of academic interest but perhaps also of some practical importance to decide whether the inhibition of pharmacological activity exercised by the one drug on the other extends also to an inhibition of therapeutic efficacy. Investigation of this aspect is not yet complete but is sufficiently important to warrant speculation at the present stage.

Chemotherapeutic interference between these compounds is, on the whole, unlikely. The pharmacological responses we have studied have all been of the kind that come into effect almost immediately after injection while the pentamidine is present in the system during the very short period of its maximum concentration. This is obvious enough in our investigations on blood pressure, bronchial and intestinal tone, and neuro-muscular transmission. It is less obvious in regard to toxicity for mice, where many of the deaths occur not immediately but some hours after the injection, though here also the result is probably largely dependent on the

impact of high concentrations of drug on vital centres immediately after the injection. The pharmacological interference we have studied occurs, therefore, during the very short period when the two compounds are present in the system in maximal concentrations and when conditions are accordingly appropriate for formation of an inactive salt complex. Chemotherapeutic activity, on the other hand, comes into play when either compound is present not only at much lower concentrations than are necessary for the pharmacological effects studied, but also during a very much longer period of exposure of the reactive tissue (in this case the trypanosome cell) to drug. Trypanosomes may not disappear from the blood stream for several days after a curative dose of either suramin or pentamidine, and Wormall and his colleagues (Town *et al.*, 1950) have produced strong evidence for the view that, after an injection, suramin is gradually released from drug-protein complexes in the plasma and may then enter the trypanosome in amounts which would be slightly inhibitory over a long period of time. Quite a low grade of inhibitory enzyme action, if maintained sufficiently long, could so disorganize the trypanosome's metabolism as to lead to its death and fully account for the therapeutic effect. Pentamidine has been less studied from these points of view, but it also is known to act relatively slowly, and the antitrypanosomal action of both suramin and pentamidine is known to persist for a very long time after an injection, as testified by a protective period of several months after the injection of a single prophylactic dose of either compound. Under these conditions of relatively slow action over a long period of time in low concentrations, complete dissociation of the suramin-pentamidine salt complex is to be expected, and each compound should exercise its action on the trypanosome unobstructed by the other.

This work therefore provides a basis of evidence, and of theory which yet requires further experimental support, for a unique situation in therapeutics where two compounds tend to annul one another's pharmacological or toxic effects while acting in concert (if not synergistically) as chemotherapeutic agents.

## Summary

1. The chance observation that a precipitate is liable to form in mixtures of dilute solutions of suramin and pentamidine raised the question whether the simultaneous presence of these two substances in the body might tend to inhibit the pharmacological effects of either of them. This has been substantiated by showing that a previous injection of suramin (or the presence of suramin) can inhibit the following actions of pentamidine: fall of blood pressure, broncho-constriction, contraction of gut, " curare-like " action on the rat phrenic nerve diaphragm preparation, paralysis in frogs and toxicity for mice.

2. Reasons are given for attributing this inhibitory effect mainly to the formation of an inactive salt complex between the suramin and pentamidine. The salt complex may, of course, be present even when the concentrations of the component substances are outside the range of those necessary for the actual formation of a precipitate.

3. Some of the pharmacological effects of pentamidine are known to be attributable to histamine release, and we have found that suramin can exercise antihistamine activity under certain conditions. It is possible, therefore, that this antihistamine

action contributes towards suramin's inhibitory influence on pentamidine, but reasons are given for believing that it plays a secondary role in some of the examples studied and is negligible in others.

4. Theoretical considerations make it unlikely that this inhibition of the pharmacological effects of the one drug by the other embraces also an inhibition of chemotherapeutic activity.

This work was supported by grants from the Medical Research Council to E. M. L. and from the British Council to J. L. G.

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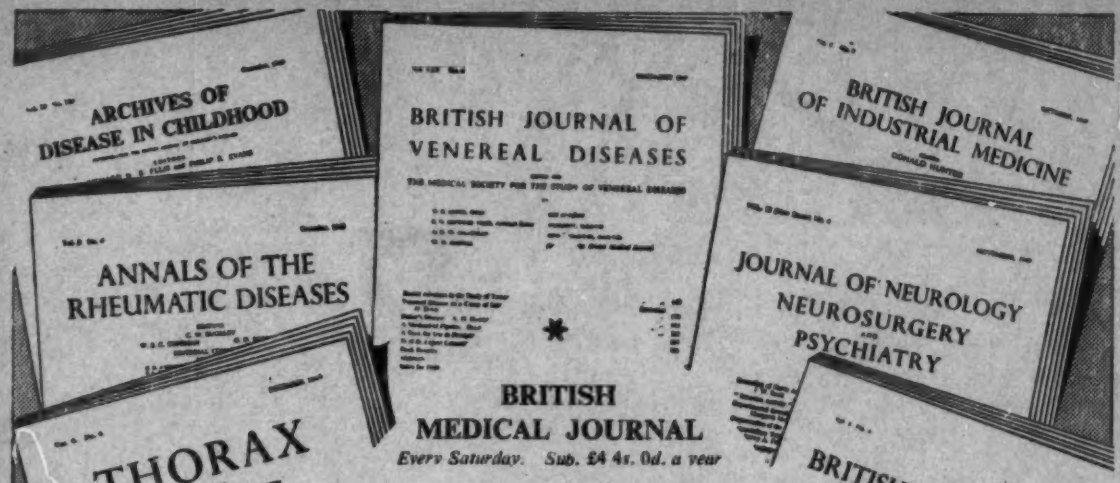
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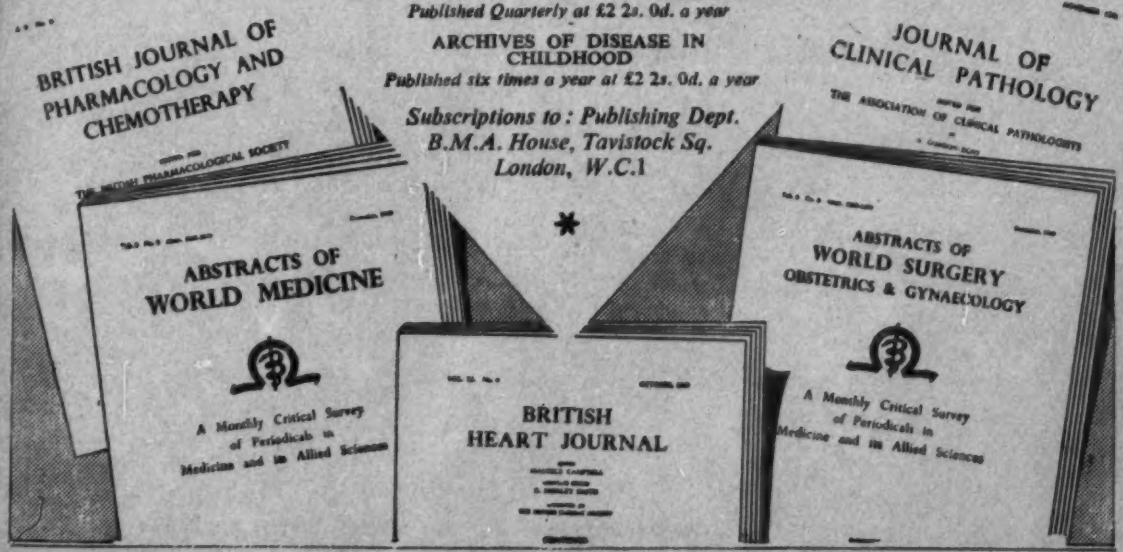
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## CONTENTS


PAGE

A Pressor Substance in Urine J. DEKANSKI.....351

Cytotoxic Agents: III, Derivatives of Ethyleneimine
J. A. HENDRY, R. F. HOMER, F. L. ROSE, and A. L. WALPOLE.....357

A Method for the Assay of Antidiuretic Activity MICHAEL GINSBURG..411

Some Observations on the Biological Properties of Bacitracins A, B, and C
G. G. F. NEWTON, E. P. ABRAHAM, H. W. FLOREY, N. SMITH, and JOAN ROSS 417

Some Biological Properties of Cephalosporin $P_1$
A. C. RITCHIE, N. SMITH, and H. W. FLOREY.....430

Prolongation of Action of Chorionic Gonadotrophin
K. DIDCOCK, J. M. ROBSON, and A. A. SHARAF.....445

Convulsive Agents and the Phosphates of Brain Examined *in Vitro*
G. ANGUIANO and H. MCILWAIN.....448

Studies in the Chemotherapy of Tuberculosis: Part VII. The Oxidation and Reduction Products of Thiosemicarbazones
E. HOGGARTH and A. R. MARTIN .....454

Action of Calabash Curare and Related Curariform Substances on the Central Nervous System of the Cat S. SALAMA and SAMSON WRIGHT.....459

The Antidiuretic Response to and Excretion of Pituitary (Posterior Lobe) Extract in Man, with Reference to the Action of Nicotine
GEORGE P. BURN and R. SINGH GREWAL.....471

The Effects of Dithiols on the Distribution of Mercury in Rabbits
K. R. ADAM .....483

The Mechanism by which N: N-Dibenzyl-Chloroethylamine Protects Animals Against Cardiac Arrhythmias Induced by Sympathomimetic Amines in Presence of Cyclopropane or Chloroform GEORGE FAWAZ.....492

Compound 48/80: A Potent Histamine Liberator W. D. M. PATON....499

The Influence of an Antihistamine Drug on the Release of Histamine in the Unanaesthetized Dog W. D. M. PATON and M. SCHACHTER.....509

The Inhibition of Some Pharmacological Actions of Pentamidine by Suramin
J. L. GUIMARAES and E. M. LOURIE.....514


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